THE INFLUENCE OF SERUM GONADOTROPIC HORMONE ON THE HISTAMINE CONTENT OF AND EASE OF RELEASE OF HISTAMINE BY TRYPSIN FROM THE GUINEA-PIG LUNG

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It has been shown previously that at birth the histamine content of the lungs of guinea-pigs is very high especially when considered on a weight basis (Trethewie, 1947). It was also found that following the injection of trypsin, the output of histamine from the lungs of new-born guinea-pigs, when the weight of the animal was considered, is greater than from perfused lungs of guinea-pigs 10–11 weeks old (Trethewie, 1949). While the increased responsiveness to injury in new-born animals may be due to the high histamine content of the lungs, it was thought worth while to determine if hormone circulating in higher concentration in the blood at term might be an important factor. It was therefore desired to determine if serum gonadotropin exerted an influence on the content of histamine in the lung and the ease of release of histamine by trypsin, especially in view of the effect of peptone and other factors (Trethewie and Day, 1948; Trethewie, 1949).

While it was found that there was a greater histamine content (22 p.c.) in the lungs of treated animals, the output of histamine from the lungs was the same in each instance. There was also inhibition of the response to some of the histamine released from the treated animals' lungs. This inhibitory effect was presumably due to a substance which could be adsorbed by alumina.

METHOD.

Selection of Animals.

Forty-seven new-born guinea-pigs were obtained from a constant breeding source. The animals were randomized. The control animals were tagged on the left ears and the animals to be treated were tagged on the right ears. At the age of two weeks the control guinea-pigs were given 0·05 ml. saline and the treated guinea-pigs 0·05 ml. of Serogan (10 I.U. of a preparation of follicle-stimulating hormone from the serum of pregnant mares). Injections were made subcutaneously. After this injection the animals were placed in a separate pen. Altogether eight injections were given to each guinea-pig at intervals of one week. Through-

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out this paper the two groups will be referred to as control and "Serogan" groups respectively. Within a week of the last injection, the lungs of the survivals of Nos. 1—24 and 28 and 30 were perfused with Tyrode. The toxicity of histamine was determined on the remaining nineteen animals. All animals were between 10 and 11 weeks of age when the assay of histamine in the lung or the toxicity of histamine was determined.

Lung Perfusion.

The animals were stunned by a light blow on the head and the lungs perfused by the method of Feldberg and Kellaway (1937 a). The perfusion rate was reduced to 0·6–0·9 ml./min. after the blood was washed out by Tyrode solution. The ventilation was 11 strokes/min. The temperature of the bath was kept constant at 36° C. throughout the perfusion. When the lobes were ventilating well, S1 perfusate was collected. After this the ventilation and the perfusion were interrupted and 0·5 ml. Difco trypsin (20 mg./ml. concentration) was injected into the arterial cannula and allowed to act for 1½ minutes. The perfusion was then re-established and S2 was collected for 30 minutes. The volume of air used for ventilation was steadily increased from the time of the injection of trypsin so that the lobes were fully distended and most of the fluid in the tissue spaces was expressed. The lungs after perfusion were cut down, placed in a small funnel, gently squeezed and allowed to drain for 30 minutes. After collection, S1 and S2 samples and the drainage fluid were all immediately boiled. The lungs were then squeezed repeatedly between filter papers to remove excess fluid. The lungs were weighed, cut finely with the scissors and extracted with Tyrode and sand. This extract was boiled. The boiled sample of S2 was usually divided into two parts and one adsorbed with Al_2O_3 (Anrep, Ayadi, Barsoum, Smith and Talaat, 1944). In some cases a portion of the drainage fluid was adsorbed also.

Histamine Assay.

Histamine was estimated as histamine acid phosphate on the isolated jejunum (guinea-pig) suspended in Tyrode.

A further test was carried out on the histamine content of the lung after the addition of neo-antergan (N-dimethyl amino ethyl N-methoxy benzyl α amino pyridine) to the bath. An estimation was also made on the blood pressure of an atropinized vagotomized cat.

Toxicity of Histamine.

The remaining animals were injected subcutaneously with 10·23 mg. histamine/kg. and the death times noted.

EXPERIMENTAL.

The Influence of Serogan on the Histamine Content of the Lung:

In twenty-two instances the histamine content of the lungs of guinea-pigs was estimated. Eleven animals belonged to the "Serogan" series and eleven were controls. The histamine content of the lungs of the "Serogan" group varied from 24–118 μg./gm. with an average of 72. In the control group the histamine contents varied from 26–100 μg./gm. with an average of 59 (Table 1). A comparison of the gut assay with the blood pressure assay of a cat was made. The concentration of histamine in the lung of guinea-pig 8 was estimated as 32 μg./gm. by the gut assay and as 33 μg./gm. by the blood pressure method. Further it has been shown (Feldberg and Kellaway, 1937 a, b) that the immediate contraction of the gut produced by such an extract is due to histamine.

TABLE 1.

Details of all experiments.

No. animal	Conc. H. in S2.	Vol. of S2.	Conc. H. in S2 (ads.)	Conc. H. in D.	Vol. of D.	µg. H. in S2.	µg. H. in S2. (ads.)	µg. H. in D.	Total output H. (µg.)	µg./gm. H. in lung	Wt. lung (gm.)	Total H. in lung (µg.)	Weight animal (gm.)	H. in µg/gm. animal
Treated:														
4	1/8·6 M.	21·5	Not titrated	1/8 M.	1·4	2·50	Not titrated	0·18	2·68	80	2·65	212	468	0·45
6	1/18 M.	23·2	Not titrated	1/10 M.	1·2	1·29	Not titrated	0·12	1·42	118	2·58	304	505	0·60
7	1/34 M.	23·0	1/25 M.	1/4 M.	1·6	0·70	0·92	0·40	1·10	24	2·69	64·6	490	0·13
8	1/60 M.	23·6	1/8·6 M.	1/7·3 M.	1·8	0·39	2·74	0·25	0·65	32	2·05	66	388	0·17
10	Nil	29·0	1/60 M.	1/4 M.	2·0	Nil	0·48	0·50	1·30	50	2·76	138	483	0·29
13	1/30 M.	24·0	1/30 M.	1/20 M.	1·0	0·80	0·80	0·05	0·85	111	2·10	233	440	0·53
16	1/59 M.	23·0	1/30 M.	1/13 M.	1·4	0·39	0·77	0·11	0·51	100	2·15	215	372	0·58
18	1/12 M.	21·4	1/8 M.	1/3·1 M.	0·7	1·78	2·68	0·22	2·02	91	2·08	189	430	0·44
24	1/33 M.	23·2	1/20 M.	1/20 M.	2·6	0·70	1·17	0·13	0·83	70	2·49	174	368	0·47
28	1/20 M.	18·5	1/13 M.	1/2·2 M.	1·6	0·93	1·40	0·73	1·66	75	2·23	167	398	0·42
30	1/4 M.	19·2	1/2·5 M.	1/3·4 M.	1·8	4·80	7·70	0·53	5·33	40	2·31	92	395	0·23
									Av. 1·67	Av. 72		Av 169	Av. 430	Av. 0·39
Control:														
3	1/6·7 M.	24·5	Not titrated	1/4 M.	1·4	3·65	Not titrated	0·35	4·05	63	2·45	154	548	0·28
5	1/18 M.	26·5	Not titrated	1/10 M.	1·0	1·47	Not titrated	0·10	0·60	67	2·08	139	441	0·32
9	1/40 M.	24·0	1/40 M.	1/8 M.	1·0	0·60	0·60	0·13	0·73	26	1·99	51·7	431	0·12
12	1/100 M.	26·0	1/100 M.	Nil	1·0	0·26	0·26	Nil	0·30	66	2·65	175	470	0·39
14					Not titrated					27	2·12	57	353	0·16
15	1/9 M.	18·0	1/11 M.	1/1·3 M.	2·0	2·00	1·64	1·60	3·60	100	2·00	200	310	0·65
17	1/30 M.	21·4	1/30 M.	1/37 M.	1·2	0·71	0·71	0·03	0·73	85	2·60	221	570	0·39
20					Not titrated					27	2·88	77·8	405	0·17
21	1/23 M.	20·0	Not titrated	1/2·4 M.	2·0	0·77	Not titrated	0·08	0·98	43	2·33	100	300	0·33
22	1/17 M.	26·4	1/20 M.	1/7·5 M.	1·4	1·55	1·34	0·19	1·69	100	2·74	274	496	0·55
23	1/25 M.	24·1	1/25 M.	1/4·4 M.	1·6	0·94	0·94	0·36	1·36	45	3·19	144	510	0·28
									Av. 1·67	Av. 59		Av. 145	Av. 439	Av. 0·33

Since we also determined the weight of the lungs we were able to estimate the histamine content in the whole lungs. This was 65–304 μg. in the "Serogan" series (average 169) and 52–274 μg. (average 145) in the control group. When estimated as μg./gm. animal the figure varied from 0·13 to 0·60 (average 0·39) for the "Serogan" series and from 0·12 to 0·65 (average 0·33) for the control series. These figures were the histamine contents measured after perfusion. Thus the histamine contents prior to perfusion would represent figures a little higher. Since there was a slightly greater output of histamine in the treated animals (using the figures for the samples adsorbed with aluminium oxide,) than in the controls the difference in histamine contents would be slightly increased were this correction made. However, it can only account for 0·5–1 μg. on the average and since in two instances in the control series the output of histamine following injection of trypsin was not estimated and in the first two experiments of the treated series the histamine content of the perfused sample was not determined after adsorption of the inhibitor, this correction was not made.

While the increase in the histamine content of the lung in the "Serogan" series is apparently sizeable, being 72 μg./gm. in the treated animals as compared with 59 μg./gm. in the controls (an increase of 22 p.c.), this difference is not significant statistically. The increase in the histamine content of the lungs is similar to that of the peptone-treated animals compared with their controls (Trethewie and Day, 1948).

TABLE 2.

Number of animals in each group obtained from Table 1.

	Range (μg.)	Control	"Serogan"
Total histamine content of lungs as μg.	50–180	8	6
	180–310	3	5
Total histamine as μg./gm. animal	0·1–0·4	9	4
	0·4–0·7	2	7
Histamine as μg./gm. lung	20–70	8	4
	70–120	3	7

When the animals are classed into two groups by dividing them into the upper and lower halves according to the histamine content of their lungs, we obtain the Table above (Table 2). Of the eleven animals in the treated series four are in the lower group and seven in the upper group, and of the eleven animals of the controls, eight are in the lower group and only three in the upper. Statistical analysis shows that this difference (binomial theorem) is not significant ($p = 0·08$). When the histamine contents are compared as μg./gm. animal, we find that nine animals give a figure within the limits of 0·1–0·4 and two animals give figures corresponding to 0·4–0·7 in the controls. In the "Serogan" animals four are in the lower and seven in the upper group. This difference is significant ($p = 0·04$). This corresponds to the similar findings in the case of animals subjected to peptone treatment when compared with their controls investigated earlier (Trethewie and Day, 1948). This means that there is a significantly greater number of animals with a high histamine content of the lungs/gm. animal following "Serogan" treatment.

This finding may only be an expression of an injurious effect of the Serogan extract due to impurities present and may not be due to the active principle (endocrine) present.

Output of Histamine Following Injection of Trypsin.

In all eleven instances the histamine liberated into the perfusion fluid from the perfused lungs of the "Serogan" animals was estimated. The direct assay of the boiled samples on the guinea-pig jejunum revealed that the concentration of histamine varied from nil (once) to 1:4 million. The output of histamine in μg. was 0–4·8. The output of histamine in the drainage fluid in these experiments varied from 1:20 million–1:2·2 million, and the contents varied from 0·05–0·73 μg. The total output in the perfused and drainage fluids averaged 1·67 μg. Further confirmation that the substance in the perfusates causing a rapid contraction of the gut was histamine was obtained by adding 2·5 μg. neo-antergan to the bath. Inhibition of the responses to 0·02 μg. histamine and 0·6 ml. of S2 perfusate (which contained a concentration equivalent to 1:33 million histamine) resulted.

In nine instances in the control series the output of histamine was estimated in the perfused samples obtained from the perfused lungs. It varied from 1:100–1:6·7 million giving an output of 0·26–3·65 μg. The histamine in the drainage fluid varied in concentration from nil (once)–1:1·3 million giving a histamine output of nil–1·6 μg. The average output of histamine in the perfused and drainage fluids was 1·67 μg. Thus the output of histamine when assayed directly on the jejunum using boiled samples was the same in the controls and in the "Serogan" series when using S2 unadsorbed samples in the comparison.

Presence of Inhibitor to Histamine in Perfused Sample.

It was noticed that sometimes relaxation of the gut occurred when the bath was washed out at the conclusion of the histamine contraction following the injection of the perfused sample. Sometimes, however, it occurred earlier. Anrep and workers (1944) observed that relaxation occurred when assaying urine for histamine and they were able to adsorb the "relaxant" with alumina. Therefore we have repeated this manoeuvre in our experiments. However, since the occurrence of relaxation was variable and occurred occasionally with both control and "Serogan" animals, while adsorption with alumina only resulted in an increase of the contractions to histamine in the "Serogan" group, we do not suggest that relaxation is produced by the inhibitor.

TABLE 3.

Comparison of the concentration of histamine in S2 before and after adsorption with Al_2O_3 in both groups.

Control series			"Serogan" series		
No. animal	Conc. of histamine in S2 (unads.) μg./ml.	Conc. of histamine in S2 (ads.) μg./ml.	No. animal	Conc. of histamine in S2 (unads.) μg./ml.	Conc. of histamine in S2 (ads.) μg./ml.
9	0·025	0·025	7	0·03	0·04
12	0·01	0·01	8	0·017	0·116
15	0·111	0·091	10	0·00	0·05
17	0·033	0·033	13	0·033	0·033
22	0·06	0·05	16	0·017	0·033
23	0·04	0·04	18	0·083	0·125
			24	0·03	0·05
			28	0·05	0·077
			30	0·25	0·4
	Av. 0·047	Av. 0·042		Av. 0·057	Av. 0·103

The findings from the assays before and after adsorption are shown in Table 3. It is noted that in the control group there is no significant difference in the assay comparing the adsorbed and unadsorbed S2 samples. In fact the average concentrations were 0·042 and 0·047 μg. histamine/ml. respectively. In the "Serogan" group the average concentrations were 0·103 and 0·057 μg. histamine/ml. respectively, revealing a notable difference of 0·046 μg. The average output of histamine in the perfused sample after adsorption was 2·1 μg. and the output before adsorption was 1·2 μg. This difference is significant statistically.

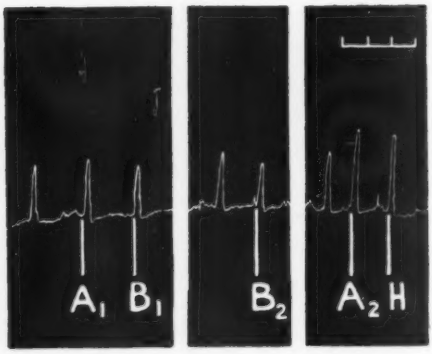

Fig. 1. Responses of the isolated jejunum of the guinea-pig. At A1 0·6 ml. S2 perfusate given (P18). At B1 0·6 ml. S2 perfusate (P22). At B2 0·6 ml. S2 adsorbed with alumina (P22). At A2 0·6 ml. S2 adsorbed with alumina (P18). At H 0·07 μg. histamine. The unlettered contractions are to 0·04 μg. histamine.

Fig. 1 shows the results of two experiments, one on a treated (No. 18) and one on an untreated guinea-pig (No. 22). At A1 and B1 are shown the responses to 0·6 ml. of the perfused samples obtained after the injection of trypsin from a "treated" and a "control" lung respectively. At A2 it is noted that the response to the same volume of the sample from the "treated" lung, when adsorbed, is increased significantly, whereas that in the case of the "control" lung (B2) produces, if anything, a slightly reduced response. The difference in the histamine contractions is considerable in the treated animals, whereas any difference in the contractions in the control animal experiments is negligible.

Absence of Inhibitor in the Serogan Preparation.

Eleven I.U. Serogan were added to a solution of histamine yielding a final concentration of 1:2 million. The contraction caused by 0·2 ml. of this solution was slightly greater than the contraction produced by 0·1 μg. histamine. Thus there was no inhibition of the response of the gut to histamine when this amount of Serogan was added.

Toxicity of Histamine.

The remaining nineteen animals (ten "Serogan" animals and nine control animals) were used to estimate any change in toxicity due to the Serogan treatment. The animals were 70–72 days old. 10·23 mg. histamine/kg. of animal at a concentration of 10 mg./ml. were injected subcutaneously on the abdomen. This is more than twice the $l.d._{50}$ for animals of this size (Trethewie, 1949). Death times were recorded in minutes and the survivals were observed for 48 hours and then they were sacrificed.

The average death time of the control animals was 18·7 minutes and of the "Serogan" animals 34·5 minutes. This difference is highly significant statistically. Two treated animals survived the treatment but no untreated animals survived. Thus in spite of the use of a very large dose of histamine (more than 100 p.c. increase of the $l.d._{50}$ for animals of the same age and stock), the treated animals were able to withstand this dose for a considerable time. In fact they survived almost to the limit of time at which death can occur after the subcutaneous injection of the quickly and evanescently acting histamine and actually two animals recovered.

TABLE 4.

Toxicity of 10·23 mg/kg. histamine.

Control series		"Serogan" series	
Death times (min.)	No. of deaths / Total No. animals	Death times (min.)	No. of deaths / Total No. animals
15		28	
36		41	
24		41	
12		41	
18	9/9	23	8/10
15	i.e. 100 p.c. mortality	20	i.e. 80 p.c. mortality
7		58	
17		24	
24			
Av. 19 min.		Av. 35 min.	

DISCUSSION.

It has been found that the histamine content of the lungs of new-born guinea-pigs is extremely high and the standard deviation is small. At twenty weeks the standard deviation is increased to twice that at eight weeks (Trethewie, 1947). The histamine content may be a function of some internal factor. As the animals grow they are also exposed to stimuli from the environment, some to more stimuli than others, so that the variation in the histamine content may depend also on outside factors. Since peptone injections did not increase the histamine content of the lungs greatly (Trethewie and Day, 1948) we have endeavoured to determine if an internal factor (endocrine) might operate.

We have found in these experiments that while there is not a considerable increase in the histamine content of the lungs of Serogan injected animals (only 22 p.c.), a significantly greater number of test animals than control animals had a high histamine content (upper half of range) in the lungs in relation to the size of the guinea-pig. From the previous work (Trethewie and Day, 1948) we were therefore led to suspect that due to the stimulation from the environment, the histamine content is normally raised, more in some animals than in others, depending on the number of stimulations and ability to respond. The treatment with Serogan may have enhanced the action of the internal factor normally present so that a greater number of test animals than control animals are in the upper group. On the other hand this may have been due to impurities in the Serogan producing a "peptone-like" action.

In our experiments we could find no great difference in the output of histamine from the perfused lungs following trypsin injections between the two

groups. This may be due to stimulation from the environment causing a more marked responsiveness to trypsin, another injurious agent, in each group of animals; and hence the effect of the Serogan is masked. On the other hand, there was a significantly greater output of histamine, as estimated after alumina adsorption, from the lungs of the treated guinea-pigs than was the case after estimation without adsorption and even this latter amount was slightly greater than either output of histamine from controls.

The difference in death times of the treated and control animals is highly significant statistically. If a smaller dose had been used this would probably have expressed itself in a greater difference in the number of survivals. Even with this dose, i.e. more than twice the $l.d._{50}$ (Trethewie, 1949) two out of 10 animals in the "Serogan" group survived. It would appear therefore that an increased resistance to the toxic effects of histamine is brought about by the Serogan injections, possibly due to the formation of inhibitor substance. This may indicate that a physiological action of the F.S.H. of the anterior pituitary is valuable in reducing the detrimental effects of certain injurious stimuli and that it acts particularly at birth.

A substance inhibitory to the histamine contractions of the gut appeared to be released by the action of trypsin from the perfused lungs of the treated animals. We were able to remove it by adsorption with alumina. It was not identical with the relaxing substance released into the perfusate from treated and untreated animal lungs. This was evidenced by the fact that perfusate from control lungs which produced a relaxation did not show an increased content of histamine following adsorption. The effects of the relaxant are particularly noticed when the amount of histamine in the perfused sample is small. It was found that only some guts responded to the relaxing substance. Also, the sensitivity of each gut varied from time to time. Inhibitor was not found in the Serogan preparation in a dose equivalent to that present in lung extract. It was shown that the amount of contraction corresponding to a solution of 0·1 μg. histamine containing 11 I.U. Serogan (a greater amount than could have been present in the lung perfusate) was not reduced; if anything, it was slightly increased.

SUMMARY.

The total content of histamine in the lungs was somewhat (but not significantly) higher in guinea-pigs treated with Serogan than in control animals. When the amount of histamine in the lungs was estimated and the weight of the animal accounted for, there was a significantly greater number of "Serogan" animals in the upper half of the range than the number of control animals within the same limits.

No significant difference was detected in the total output of histamine between the two groups following the injection of trypsin.

The presence of an inhibitor to histamine was discovered in the perfused sample from the lungs of the "Serogan" group.

The toxicity to histamine appeared to be reduced by the Serogan treatment.

The possible significance of these findings is discussed.

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THE INFLUENCE OF CROP ROTATION ON THE THIAMIN RIBOFLAVIN AND NITROGEN CONTENTS OF WHEAT[1]

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In an earlier investigation Clare and Underwood (1948) determined the thiamin and riboflavin contents of wheat and its mill products from mills in Western Australia. The riboflavin content of the wheat grain was found to be very similar to that obtained by Morell (1947) for other parts of Australia, and confirmed his finding that the average riboflavin content is appreciably lower than that obtained for wheat in most overseas investigations. The thiamin content of Western Australian wheat (and flour) was also found to be within the ranges found in earlier investigations on an Australia-wide basis, although the values definitely fell into the lower parts of the ranges. These findings indicated the need for further study of the factors affecting the thiamin and riboflavin contents of wheat. For a variety of reasons the influence of crop rotation was felt to be worthy of immediate investigation.

No reports on the effects of crop rotations on the amounts of these two vitamins in wheat grain are available, although some information on the effects of soil treatment on the thiamin content was obtained by Harris (1934) and Leong (1939), both using the bradycardia method of assay. These workers found no great change in thiamin content with different soil treatments, although the former did observe some increase when ammonium sulphate was used as fertilizer. Also in work carried out by the Nutrition Committee of the National Health and Medical Research Council (1942) it was found that a sample of wheat grown on "old" land in 1940–41 contained considerably less thiamin than the same variety from "old" land which had been under a legume (clover) for 10 years. A similar difference was not obtained in samples obtained from the same sources in the following year.

Two quite independent types of investigation have, however, conspired to focus attention on the importance of crop rotation as a possible factor affecting the thiamin content of wheat, although it must be mentioned that no such evidence has been obtained in the case of riboflavin. These are, firstly, the finding by several workers that there is a significant positive correlation between the protein and thiamin contents of wheat grain, and, secondly, that the inclu-

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sion of a legume in the crop rotation increases the protein (gluten) content of the grain. The former was reported by Connor and Straub (1941), Hoffer, Alcock and Geddes (1944) and Jackson and Whiteside (1943) in North America, and also by the Nutrition Committee of the N.H.M.R.C., mentioned above. In this earlier investigation, the thiamin-protein correlation for Australian wheat was + 0·64 in the 1940–41 season, and + 0·45 in the 1941–42 season. This finding is supported further by the figures given by Moran and Jones (1946) for the variation in average thiamin content and average protein content for the entire crops of different seasons. The value of a legume in the rotation in increasing the protein content of wheat grain was found by Samuel (1945) and Shier and Cullinane (1948) from a study of crop rotation experiments in Western Australia, in which protein (gluten) contents and flour strengths, as well as yields, of grain were obtained.

In this paper the results of a study of the thiamin, riboflavin and nitrogen contents of wheat from rotation experiments in Western Australia for the five-year period 1944–48 and from a rotation experiment in South Australia for the four-year period of 1945–48 are presented.

Materials and Methods.

Samples.

The wheat samples were collected from rotation trials conducted by the Western Australian Department of Agriculture at their Research Stations at Chapman, situated near the northern limit of the wheatbelt, at Wongan Hills, situated about 120 miles north-east of Perth, and at Merredin, situated about 160 miles east of Perth, near the eastern limit of the wheatbelt. Unfortunately, it was only possible to obtain one (1945) set of samples from Merredin, as the experiment was then discontinued. At both Chapman and Wongan Hills the soils on which the rotation experiments were located were "light," the former a brown to red-brown sandy loam at the surface, with rather more clay in the subsoil, and the latter a sandy loam, typical of large areas of laterite "sand-plains" in Western Australia.

The rotations at these two stations comprised:

1. Continuous wheat.
2. Fallow, wheat.
3. Stubble, fallow, wheat.
4. Lupins, lupins, fallow, wheat.

The layout was duplicated, so that two plots of each rotation were sown to wheat each year. The samples analysed represented a mixture of these two plots for each treatment in each year and were obtained for the five-year period 1944–48 inclusive. 60 lb. of seed per acre were used at Chapman and 45 lb. per acre at Wongan Hills. Superphosphate was the only fertilizer used and was applied with each wheat crop. The variety planted varied from year to year as follows:

Chapman: 1944 and 1945, Nabawa; 1946, Bungulla; 1947 and 1948, Eureka.
Wongan Hills: 1944, Merredin; 1945 and 1946, Koorda; 1947 and 1948, Charter.

The relatively "strong" varieties (Eureka and Charter) were introduced in 1947 in order to obtain a greater range of flour strengths in the experiments.

Samples of wheat were also obtained from a Rotation Trial, rather different in design, carried out at the Waite Agricultural Research Institute, Adelaide, South Australia. Nine different rotations were included in this experiment as follows:

A1 Fallow, wheat, fallow, wheat.
A2 Fallow, wheat, fallow, wheat.
B1 Fallow, wheat, barley grazed, pasture.
B2 Fallow, wheat, barley harvested, pasture.
C1 Fallow, wheat, oats grazed, pasture.
C2 Fallow, wheat, oats harvested, pasture.
D1 Fallow, wheat, clover grazed, pasture.
D2 Fallow, wheat, clover harvested, pasture.
E1 Fallow, wheat, peas grazed, pasture.
E2 Fallow, wheat, peas harvested, pasture.

Each rotation was carried out on three separate plots so that 30 samples of wheat were obtained in each year. In 1945 all 30 samples were analysed separately. In 1946, 1947, and 1948 samples of the grain from the three plots of the various treatments were thoroughly mixed and aliquots taken for analysis. Unfortunately, it was not possible to obtain samples from each treatment in these three years. In 1946 and 1947, treatments A1, A2, C1, C2, E1 and E2 only were sampled, and in 1948 treatments A1, A2, B1, B2, C1, C2, D1, D2, E1 and E2.

It should be noted that this experiment differed in certain respects from those at Chapman and Wongan Hills. In the first place, the soil type was heavier. The soil at the Waite Institute, on which this experiment was located, is a red-brown fine sandy loam, overlying red, friable clay. In the second place, the legume included in rotations D1, D2, E1 and E2 was sown in one year only, compared with two years for the lupins in the Western Australian experiments. This point will be referred to later. Superphosphate at the rate of 112 lb. per acre, was the only fertilizer applied and seeding of the wheat was at the rate of 81–85 lb. per acre. The varieties of wheat employed were Sepoy in 1945, Seewari in 1946 and 1947 and Seewari 48 in 1948.

Methods of Analysis.

Thiamin.

The method used was essentially that of Slater (1946). This is an improved version of the earlier method of Slater (1941), who adapted the thiochrome method of Wang and Harris (1939) for use on cereals and cereal products. Our method differed slightly from the improved method of Slater, in that the method of addition of reagents was that described by Hinton (1943). Readings were made with "Spekker" fluorimeter using a "Woods Glass" ultraviolet filter between the lamp and the fluorescent solution and a Wratten 39 and Wratten 2A between the solution and the photocell.

Riboflavin.

The highly specific method of Slater and Morell (1946) which was based on the method of Najjar (1941) was used. The extraction procedure used involved autoclaving the sample with dilute hydrochloric acid followed by buffering to pH 4·5 and incubation with papain and takadiastase. This extraction procedure is described in detail by Morell (1947). Fluorescence was read in a Spekker fluorimeter using a Wratten 39 filter between the light and the fluorescent solution and Wratten 8 and Wratten 2A filters between the solution and the photocell.

Nitrogen.

The normal Kjeldahl method was used with selenium as catalyst. The digestion time, after clearing, was 2 hours. The ammonia was distilled into 4 p.c. boric acid and directly titrated with standardized HCl with methyl red-bromphenol blue indicator.

RESULTS.

The individual results for each treatment, in each year at each location are given in an appendix. These are not readily amenable to statistical analysis, and it is necessary to stress the fact that the varieties of wheat varied from year to year, both within and between experiments. The environmental conditions at each location also differed greatly. For convenience of discussion, however, the data are presented in a summarized form in Tables 1, 2, and 3. In these Tables the average nitrogen, thiamin, and riboflavin values for the various treatments together with the average yields of wheat in bushels per acre over the same period are given. The yield figures are included as a matter of considerable interest and importance since the primary objective of the experiments was to determine effects on yields.

TABLE 1.

Average nitrogen, thiamin and riboflavin contents of wheat
(For the 5-year period 1944–48, expressed on dry basis).

Treatment	Wongan Hills				Chapman			
	Bushels* per acre	Nitrogen p.c.	Thiamin µg/gm.	Riboflavin µg/gm.	Bushels* per acre	Nitrogen p.c.	Thiamin µg/gm.	Riboflavin µg/gm.
Continuous wheat	4·4	1·58	4·0	0·9	3·9	2·05	5·0	0·9
Fallow, wheat	13·6	1·73	4·3	1·0	6·5	1·63	4·5	1·0
Stubble, fallow, wheat	17·6	1·71	4·1	1·0	8·3	1·48	4·2	1·0
Lupins 2 yrs., fallow, wheat	20·3	1·87	4·4	1·0	13·6	2·01	5·1	1·1

* Figures kindly supplied by Mr. F. L. Shier, of the Western Australian Department of Agriculture.

Effect of Crop Rotation.

It is apparent from Tables 1 and 3 that there is no obvious relationship between the riboflavin content of wheat and the rotation in which the wheat is grown. At Merredin in 1945 (Table 2) the wheat from the longer rotations is higher in riboflavin content than that from the shorter. Too much significance cannot be attributed to this result, however, in view of the fact that the data are for one year only, and are not supported by the data from the other research stations, based on several years' results. Also, it should be pointed out that the variation in the riboflavin content of samples from duplicate plots was, in many cases, greater than the differences between samples from different rotations. No satisfactory

explanation of this variability can be offered, although it can be stated that the method of assay is not at fault.

TABLE 2.

Nitrogen, thiamin and riboflavin contents of wheat at Merredin.

(For the year 1945, expressed on dry basis).
(Variety: Bencubbin).

Treatment	Nitrogen p.c.	Thiamin µg/gm.	Riboflavin µg/gm.
Continuous wheat	2·09	5·1	0·7
Fallow, wheat	2·55	5·4	0·8
Stubble, fallow, wheat	3·00	6·1	1·1
Renovating crops* 2 years, fallow, wheat	2·94	7·1	1·0

* Natural grasses, clovers and medics.

The nitrogen and thiamin results, however, reveal that there are changes in the content of these substances which can be attributed, with some confidence, to the rotation. At Wongan Hills, the average nitrogen and thiamin contents of the wheat for the five-year period are slightly greater for the rotation including a legume than for any other rotation. The differences are very small, but reference to the appendix shows that they occur in four of the five years studied. The results from Chapman are similar, except for the continuous wheat rotation in which both the nitrogen and thiamin contents are higher than might be expected. This is possibly due to the fact that the continuous wheat plots at Chapman have become infested with weeds, including appreciable amounts of small clovers and medics. The Merredin results (Table 2) for nitrogen and thiamin reveal rather more marked differences, although, as pointed out previously, these are unfortunately for one year only. Nevertheless, there is an appreciable increase in the nitrogen content of the wheat from both the three and four-year rotations compared with either the continuous wheat or fallow, wheat rotations, and a progressive increase in thiamin content with length of rotation.

TABLE 3.

Average nitrogen, thiamin and riboflavin contents of wheat at Waite Institute.
(For the 4-year period 1945–48, expressed on dry basis).

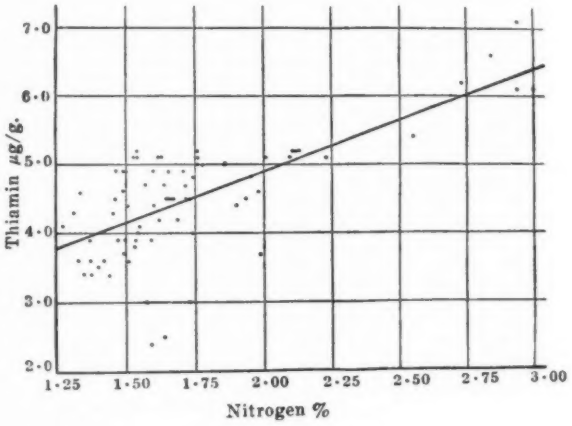
	Treatment	Bushels* per acre	Nitrogen p.c.	Thiamin µg./gm.	Riboflavin µg./gm.
A1	Fallow, wheat, fallow, wheat	23·8	1·64	4·2	1·1
A2	Fallow, wheat, fallow, wheat	22·7	1·60	4·3	1·0
B1	Fallow, wheat, barley grazed, pasture	28·2	1·55	4·3	1·0
B2	Fallow, wheat, barley harvested, pasture	27·0	1·56	4·2	1·0
C1	Fallow, wheat, oats grazed, pasture	28·2	1·52	4·3	1·0
C2	Fallow, wheat, oats harvested, pasture	26·3	1·48	4·3	1·2
D1	Fallow, wheat, clover grazed, pasture	32·6	1·53	4·3	1·1
D2	Fallow, wheat, clover harvested, pasture	32·1	1·53	4·3	1·1
E1	Fallow, wheat, peas grazed, pasture	39·7	1·75	4·8	1·0
E2	Fallow, wheat, peas harvested, pasture	34·9	1·70	4·6	1·0

* Figures kindly supplied by Mr. K. Woodroffe, of the Waite Agricultural Research Institute.

The results from the Waite Institute Trial (Table 3) reveal no appreciable effect of rotation on the nitrogen and thiamin contents of the wheat, other than in the final rotations E1 and E2, which include a year of peas either grazed or harvested. The mean nitrogen and thiamin figures from this rotation are both somewhat higher than those from the other rotations, including D1 and D2 which contain a legume, subterranean clover. In comparing these results with those given in Tables 1 and 2, it should be noted that the Western Australian experiments contained a rotation which included *two years* of legumes in succession, whereas the Waite Institute experiment was designed in such a way that there was only one year of legume growth between successive wheat crops in the final rotation. This is probably of some importance, particularly in the case of D1 and D2, containing subterranean clover. Actual yields of dry matter showed that this legume was considerably less productive, over the period 1945–48, than the peas.

Nitrogen-Thiamin Correlation.

The whole of the figures for nitrogen and thiamin from the four research stations, as presented in the appendix, were examined to determine the correlation between nitrogen and thiamin contents. For the degrees of freedom present, a highly significant positive correlation ($r = 0{\cdot}702$) was found. The proportion of the variability accounted for, as measured by the coefficient of determination, is about 50 per cent. ($r^2 = 0{\cdot}493$).



The nitrogen-thiamin scatter diagram is given in Fig. 1. The linear regression line is drawn using the calculated equation $y = 1{\cdot}88 + 1{\cdot}51x$.

Nitrogen-Riboflavin Correlation.

The correlation between nitrogen and riboflavin was also determined on the whole of the figures presented in the appendix. No significant correlation was found.

DISCUSSION.

During the last few years, agronomists and others have stressed the necessity for lengthening the rotation in the wheat belt of southern Australia, in order to arrest the decline in wheat yields which is occurring. The marked influence of such lengthening of the rotation on yields of wheat, at least for the lighter soils of Western Australia, is clearly demonstrated by the figures given in Table 1, taken from the data of Shier and Cullinane (1948), and in Table 3 from the Waite Institute Trial. Shier and Cullinane also showed that an

equally important, though much less spectacular, increase occurred in the "dry gluten" content and "flour strength" of the wheat from the longer rotations, particularly where these included two years of a legume (lupins). There appears to be a similar, though very much smaller, improvement in the thiamin content of wheat from these rotations. In the Waite Institute experiment this is only discernible in the rotations which included one year of peas. The absence of any measurable effect on either the nitrogen or thiamin content of the wheat from the rotations which included clover is probably due, as mentioned earlier, to the smaller growth made by this crop, compared with the more productive pea crop.

This raises the important question of the effect of much longer rotations, including a number of years of legume growth, on the nitrogen and thiamin contents of the wheat grain. It must be emphasized that the present experiments contain only one or two years of a legume, and the longest rotation studied is a four-year one, in which wheat crops are grown every fourth year. With the development of more productive pasture legumes for the wheat areas, and already where early strains of subterranean clover fulfil this rôle, rotations including 4–10 years of clover pasture appear desirable. How far the increasing nitrogen status of the soil which would probably result from this treatment is likely to induce further marked increases in yield, or further improvement in the nitrogen and thiamin contents of the wheat grain, remains to be determined. Some slight evidence that this occurs with longer rotations is already available in the case of nitrogen. The position with respect to thiamin is under investigation at the present time.

SUMMARY.

The nitrogen, thiamin and riboflavin contents of wheat samples, from crop rotation experiments in Western Australia and South Australia, have been determined for the period 1944–48.

Small increases in the average nitrogen and thiamin contents of the wheat from the rotations which included one or two years of a crop legume were found. Longer periods under a legume were not included in the rotations studied.

Considerable variation in the riboflavin content of the wheat samples was found, but no obvious relationship between riboflavin content and the rotation in which the wheat was grown was discernible.

A highly significant positive correlation ($r = 0{\cdot}702$) was found between nitrogen and thiamin contents of the wheat samples, but no significant correlation between nitrogen and riboflavin, or thiamin and riboflavin.

In the relatively short rotations studied (maximum four years) the effect of the legume on yield of wheat is very much greater than the effect on nitrogen and thiamin contents. The possible influence of much longer rotations, including a number of years of legume pasture, is discussed.

Acknowledgments. It is a pleasure to record our indebtedness to Mr. E. C. Slater and Mr. D. B. Morell, of the Institute of Anatomy, Canberra, for much practical help and advice in the assay procedures; to Professor Sir Stanton Hicks, of the University of Adelaide, and Professor D. H. K. Lee, of the University of Queensland, for the loan of valuable instruments during the early stages of the work, and to Mr. I. Thomas, Department of Agriculture, Western Australia, and Professor J. A. Prescott, Waite Institute, S.A., for their co-operation in supplying the wheat samples.

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APPENDIX.

WONGAN HILLS.

Variety: Merredin—1944; Koorda—1945 and 1946; Charter—1947 and 1948.

Rotation	Nitrogen p.c. dry weight					Thiamin μg/gm. dry weight					Riboflavin μg/gm. dry weight				
	1944	1945	1946	1947	1948	1944	1945	1946	1947	1948	1944	1945	1946	1947	1948
Continuous wheat	1·34	1·59	1·67	2·00	1·31	4·6	2·4	4·5	4·0	4·3	1·3	0·8	0·7	0·9	0·8
Fallow, wheat	1·67	1·64	1·76	1·86	1·72	4·5	2·5	5·1	5·0	4·5	1·3	0·8	1·0	0·9	0·9
Stubble, fallow, wheat	1·95	1·57	1·68	1·74	1·60	4·8	3·0	4·2	4·0	4·4	1·2	0·9	1·1	1·0	1·0
2 yrs. Lupins, fallow, wheat	2·22	1·72	1·71	1·97	1·74	5·1	3·0	4·7	4·6	4·8	1·1	0·9	0·9	1·1	0·8

CHAPMAN.

Variety: Nabawa—1944 and 1945; Bungulla—1946; Eureka—1947 and 1948.

Continuous wheat	1·51	1·54	1·54	2·73	2·94	3·6	4·0	5·1	6·2	6·1	0·9	0·8	1·0	0·9	1·0
Fallow, wheat	1·45	1·37	1·50	2·11	1·73	4·3	3·9	4·9	5·2	4·5	0·8	1·0	1·1	0·9	1·1
Stubble, fallow, wheat	1·53	1·40	1·27	1·53	1·65	3·8	3·5	4·1	5·1	4·5	0·9	1·1	1·1	1·0	1·0
2 yrs. Lupins, fallow, wheat	2·11	1·47	1·51	2·13	2·84	5·2	3·9	4·4	5·2	6·6	0·9	1·1	1·1	1·2	1·1

MERREDIN.

Variety: Bencubbin.

Continuous wheat	—	2·09	—	—	—	—	5·1	—	—	—	—	0·7	—	—	—
Fallow, wheat	—	2·55	—	—	—	—	5·4	—	—	—	—	0·8	—	—	—
Stubble, fallow, wheat	—	3·00	—	—	—	—	6·1	—	—	—	—	1·1	—	—	—
Renovating crops 2 yrs., fallow, wheat	—	2·94	—	—	—	—	7·1	—	—	—	—	1·0	—	—	—

WAITE INSTITUTE ROTATION EXPERIMENT (C53).

Variety: Sepoy—1945; Seewari—1946 and 1947; Seewari 48—1948.

	Nitrogen p.c.				Thiamin μg/gm.				Riboflavin μg/gm.			
	1945	1946	1947	1948	1945	1946	1947	1948	1945	1946	1947	1948
A1 Fallow, wheat, fallow, wheat	1·49	1·59	1·60	1·90	3·7	3·9	4·9	4·4	1·1	1·1	1·5	0·9
A2 Fallow, wheat, fallow, wheat	1·42	1·53	1·54	1·93	3·6	3·9	5·2	4·5	0·9	1·1	1·1	1·0
B1 Fallow, wheat, barley grazed, pasture	1·33	—	—	1·78	3·6	—	—	5·0	1·2	—	—	0·9
B2 Fallow, wheat, barley harvested, pasture	1·37	—	—	1·76	3·4	—	—	5·0	1·0	—	—	1·0
C1 Fallow, wheat, oats grazed, pasture	1·37	1·55	1·49	1·66	3·6	4·1	4·9	4·5	1·1	0·8	1·2	1·0
C2 Fallow, wheat, oats harvested, pasture	1·35	1·46	1·49	1·64	3·4	4·5	4·6	4·7	1·2	1·4	1·4	0·9
D1 Fallow, wheat, clover grazed, pasture	1·49	—	—	1·57	3·9	—	—	4·7	1·1	—	—	1·2
D2 Fallow, wheat, clover harvested, pasture	1·44	—	—	1·63	3·4	—	—	5·1	1·2	—	—	1·0
E1 Fallow, wheat, peas grazed, pasture	1·62	2·00	1·62	1·76	4·2	4·9	5·1	5·2	1·0	0·9	1·0	1·1
E2 Fallow, wheat, peas harvested, pasture	1·98	1·66	1·46	1·71	3·7	4·9	4·9	4·9	1·1	1·1	0·7	0·9

STUDIES ON PARACOLON BACILLI

by ROSE MUSHIN

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An investigation of outbreaks of gastro-enteritis occurring in a Royal Australian Air Force (R.A.A.F.) camp in Victoria showed that paracolon bacilli were isolated from the stools of patients with greater frequency than other recognized pathogens. Cumulative evidence found in literature points to paracolon bacilli as: (1) etiological agents of gastro-enteritis (Rhodes, 1942; Stuart and Rustigan, 1943; Plass, 1947; Edwards *et al.*, 1947), (2) indicators of metabolic disturbances (Stewart, 1926; Stuart *et al.*, 1940) and (3) of no importance in the intestinal tract (Sandiford, 1935). In view of our results and the conflicting opinions listed above, it seemed of interest to follow up the general problem of the significance of this group of organisms in the intestinal flora. It was thought that a study of paracolon bacilli, isolated from cases of gastro-enteritis in the R.A.A.F. camp and from other sources, may throw some light on the problem of pathogenicity. Further, in the course of an investigation of a typhoid outbreak in a Mental Hospital, whose inmates were subject to intestinal disorders, an opportunity presented itself for the study of a possible alteration in the intestinal flora of these patients.

Accordingly this paper deals with paracolon bacilli isolated from pathological and normal stools of adults, their frequency in abnormal and normal material and their classification on the basis of biochemical reactions. In addition, a number of paracolon bacilli was examined in more detail with regard to bacteriological and clinical aspects. The results established that some strains are undoubted human pathogens and others have the ability to establish themselves in the intestinal tract without manifesting clinical symptoms.

BACTERIOLOGICAL.

Isolation of Paracolon Bacilli.

Faecal specimens from enteric cases were collected in a preservative solution of buffered 30 p.c. glycerin saline (Sachs, 1939), whereas other specimens were examined as rectal swabs. A number of media was employed for the detection of the causal organisms, the less selective being MacConkey and plain desoxycholate agar (Leifson, 1935), while the more selective ones such as SS agar, a combination of tetrathionate broth followed by SS agar, Wilson and Blair's bismuth-sulphite medium and Gregory's modification of Wilson and Blair's tellurite-rosolic acid medium (1944) were included.

TABLE 1.

Frequency of isolation of paracolon bacilli and recognized pathogens from abnormal stools and rectal swabs.

Source of specimen	Classification of cases	No. of cases	No. of examinations	No. of patients with recognised pathogens	No. of isolations of paracolon bacilli	P.c. of isolations of paracolon bacilli
R.A.A.F. camp	Acute gastro-enteritis	17	37	2 (*S. flexneri* VI)	11	30
	Convalescent, recovered	17	22	0	4	18
Mental Hospital	Enteric, carriers, history of intestinal disorders	115	134	7 (*Salm. typhi*)	52*	39*
Various	Acute and chronic gastro-enteritis	40	51	5 (3 *Salm. typhi-murium*) (1 *Salm. newport*) (1 *S. sonnei*)	26*	51*

* Denotes the inclusion of plain desoxycholate agar for these isolations.

Table 1 presents the results of bacteriological investigation with regard to the distribution of paracolon bacilli in abnormal material. The pathological cases came from the following sources: (1) R.A.A.F. camp, where *Shigella flexneri VI* was detected in the stools of two patients, (2) Mental Hospital, where *Salmonella typhi* infection was diagnosed in three clinical cases and four carriers and (3) various sources with a record of one *Shigella sonnei* and four *Salmonella isolations*. The results of this survey are of interest in centering attention on the high recovery rate of paracolon bacilli from abnormal material. It was noticed that the frequency of isolations of these organisms depended on the use of different media, e.g. the examination of cases from the Mental Hospital gave figures 34, 22 and 19 p.c. respectively on plain desoxycholate, SS and Wilson and Blair's bismuth-sulphite media. In all probability the number of paracolon isolations from the R.A.A.F. camp would have been higher with the inclusion of plain desoxycholate agar which was not used at the early stage of our investigation.

TABLE 2.

Frequency of isolation of paracolon bacilli from normal stools with the use of various media.

Medium	No. of specimens	No. of isolations of paracolon bacilli	P.c. of isolations of paracolon bacilli
Plain desoxycholate agar	159	35	22
MacConkey agar	240	32	13
SS agar	228	17	7

In view of these findings, namely the predominance of paracolon bacilli over other recognised pathogens in abnormal material, it was considered important to investigate the distribution of this group of organisms in normal stools. To this end 240 faecal specimens,

derived mostly from University students and hospital patients (non-enteric cases) were examined by culturing at first on MacConkey and SS media, while later in the survey plain desoxycholate agar was also included.

It is shown in Table 2 that the percentage of paracolon isolations from plain desoxycholate agar was much higher than from MacConkey and SS media. The specimens yielded a total of 47 paracolon strains, thus giving 20 p.c. frequency but the inclusion of desoxycholate agar in the earlier part of the survey would have increased these figures. Thus plain desoxycholate agar must be regarded as the most efficient medium in detection of paracolon strains; this was specially noticed with specimens of a low paracolon count.

A comparison of results recorded in Tables 1 and 2 shows a definite tendency toward increased incidence of paracolon bacilli in pathological material over that found in normal stools. This difference was further emphasized by the abundance of paracolon colonies on plates from clinical cases, at times a pure culture being obtained in contrast to only a few colonies of this organism yielded as a rule by the positive normal stools. Moreover, an inquiry amongst these normal individuals whose stools showed a high paracolon ratio revealed frequently a recent intestinal disturbance or some kind of alimentary complaint.

Biochemical Classification of Paracolon Bacilli and Other Tests.

A survey of recent literature on the biochemical classification of paracolon bacilli shows that two sets of criteria have been adopted, the ability to ferment saccharose and dulcite (Sevitt, 1945) and the IMViC quartet of tests (Parr, 1939; Stuart *et al.* 1943). In this work the second procedure was applied as it proved to give more reliable results. The accepted tests for indole, M.R. and V.P. reactions were used, the M.R. reading being made after 5 days' incubation at 37° C. and V.P. according to O'Meara's modification. Citrate utilization in Koser's medium was considered as positive when growth occurred at 37° C. on three successive transfers at intervals of 48 hours.

The nomenclature adopted in this paper for the classification of paracolon bacilli followed the schema suggested by Borman *et al.* (1944), incorporated in Bergey's Manual of Determinative Bacteriology (1948). Accordingly, organisms producing gas in glucose were classified as *Paracolobactrum* and were divided into three major groups, which we found convenient to split into sub-groups including a section under the heading "irregular." Besides, as no provision was made for anaerogenic motile strains, we formed a group of all these paracolon bacilli which failed to produce gas in glucose.

TABLE 3.

Biochemical classification of 122 paracolon strains.

Biochemical group	77 paracolon strains from abnormal material		45 paracolon strains from normal material	
	No.	p.c.	No.	p.c.
Paracolobactrum coliforme type I (++−−)	41	53	34	76
Paracolobactrum coliforme type II (−+−−)	6	8	2	4
Paracolobactrum intermedium type I (−+−+)	9	12	2	4
Paracolobactrum intermedium type II (++−+)	1	1	2	4
Paracolobactrum aerogenoides type I (−−++)	1	1	4	9
Paracolobactrum aerogenoides type II (+−++)	1	1	0	0
Paracolobactrum aerogenoides irregular (+−+−, −−+−, −++−)	8	10	0	0
Anaerogenic	10	13	1	2

As set out in Table 3 the majority of our cultures from abnormal and normal material was classified as *Paracolobactrum* and most strains from both sources fell into *P. coliforme* type I section.

Additional biochemical and other tests were made on 84 paracolon strains comprising 53 isolated from abnormal stools and 31 from normal. Eijkman's test was performed at 44° C., d–tartrate decomposition was determined by the method of Brown *et al.* (1924) and urea decomposition with Christensen's medium (1946).

The following points are of interest: Most paracolon strains were non-motile or sluggishly motile on first isolation but the majority became actively motile after passage through semi-solid agar. Some strains which were non-motile at 37° C. were motile at 22° C. Great irregularity was noticed in lactose fermentation, which usually took place within 3 to 28 days, but a few strains did not ferment this sugar even after prolonged incubation up to 3 months at 37° C. and 22° C. in 1 p.c. and also in 5 p.c. lactose as recommended by Kriebel (1936). After a series of subcultures paracolon bacilli usually fermented lactose in a shorter time than on primary isolation and accordingly some paracolon strains became coliform; on the other hand a few slow lactose fermenters lost their ability to ferment this sugar. A number of strains recorded at first as producing gas in lactose became anaerogenic after cultivation on artificial media and vice-versa. Eijkman's reaction was negative for all paracolon bacilli, no growth, growth or acid with little gas being recorded. Mannite, glucose and maltose were constantly fermented. Variable results were recorded with regard to fermentation of saccharose, dulcite, salicin, inosite and reactions in litmus milk, liquefaction of gelatin and H_2S production. One sub-group of *P. intermedium* type I decomposed d–tartrate. Urea was decomposed slowly (in comparison with *Proteus*) by *P. aerogenoides* strains. Complete haemolysis on human, sheep and horse blood agar was accomplished by one strain only and partial haemolysis by a few strains. Thus the above tests showed a great heterogenicity in biochemical activities of paracolon bacilli, regardless of the source of isolation, viz. from pathological or normal stools.

Of special interest were six homogenous strains forming a sub-group of *P. intermedium* type I and named for convenience paracolon *melbourne*; these organisms will be discussed in more detail under the heading "Clinical findings." With the view of identification of paracolon *melbourne* strain, the following characteristics are given: actively motile on first isolation, acidifies lactose in 3 days (on first isolation), produces acid and gas in mannite, glucose, maltose, saccharose, while dulcite, salicin and inosite are not fermented, litmus milk shows acid and clot, gelatin is not liquefied, d–tartrate is decomposed, H_2S is produced, no haemolysis of R.B.C. (sheep and horse) takes place.

Serological Reactions of Paracolon Bacilli.

Paracolon strains, 36 in number, derived from normal and abnormal material were submitted to a serological investigation, including their relationship to *Salmonella* (groups A, B, C, D, E) and *Shigella* (*S. shigae, schmitzi, sonnei, dispar, flexneri* I to VI, *boydi* I to III, D 19, P 274, *alkalescens*). In all, 57 bacterial strains and 30 somatic antisera were used for agglutination and agglutinin-absorption tests. Saline suspensions for agglutination reactions were prepared in bulk from growth of smooth strains (tested with trypaflavine) on nutrient agar in Roux bottles; the suspensions were heated at 56° C. for 30 min. and preserved with 0·2 p.c. formalin. Somatic antigens for immunization of rabbits were prepared in the form of heated (at 56° C. for 30 min.) broth cultures of non-motile and boiled cultures of motile strains. Antisera to paracolon bacilli, *Salmonella* (groups A, B, C, D, E), *S. sonnei* (smooth phase) *S. flexneri* Y, *S. dispar* and *S. alkalescens* were prepared by the writer while the other tested Shigella antisera were supplied by the Commonwealth Serum Laboratories. All agglutination experiments were carried out at 52° C. for 18 hours. In assessing relationship between various strains cross-agglutination reactions lower than 1/16th of full titre were disregarded.

TABLE 4.

Cross-agglutination reactions of heated suspensions of paracolon, Salmonella *and* Shigella *bacilli against O antisera of these organisms.*

Suspension from	No. of suspensions	O antisera prepared with	No. of O antisera	No. of agglutinating strains	P.c. of agglutinating strains
Paracolon sp.	36	Paracolon sp.	9	23	64
Paracolon sp.	36	*Salmonella*	5	7	19
Paracolon sp.	36	*Shigella*	16	16	44
Salmonella	5	Paracolon sp.	9	0	0
Shigella	16	Paracolon sp.	9	15	94

Table 4 represents results of agglutination reactions of heated (at 56° C. for 30 min.) suspensions of paracolon, *Salmonella* and *Shigella* bacilli with appropriate somatic antisera of these organisms. It was shown that serological relationship existed between members of paracolon groups from normal and pathological material. Cross-agglutination reactions were recorded between some paracolon strains from both sources and *Salmonella* antisera (groups A and C) as well as between paracolon bacilli and *Shigella* antisera (*S. flexneri* Y, IV, VI, P 274, *alkalescens*). On the other hand *Salmonella* organisms did not cross-agglutinate with the tested paracolon antisera while reactions were positive between a number of *Shigella* strains (*S. shigae, flexneri* Y, IV, D 19, *alkalescens*) and the paracolon antisera. A reaction worthy of notice was one showing that six paracolon strains cross-agglutinated to a full (1/100,000) or high titre with *S. flexneri* Y antiserum; this was proved to be due to the presence of a new thermolabile antigen, which we named β antigen (discussed in a separate paper, Mushin, 1949).

Paracolon melbourne strains proved to be serologically identical as shown by agglutinin-absorption tests. The freshly isolated cultures cross-agglutinated with somatic antiserum of *Salmonella* group C but stock cultures failed to do so.

Cross-agglutination reactions with boiled suspensions of paracolon bacilli showed substantially similar results to those obtained in Table 4 with the exception of β suspensions. The latter gave negative or low titre agglutinations in contrast to the previously recorded high titre reactions and this was due to the common β antigen being destroyed by boiling.

Serological Reactions of Paracolon Bacilli with Human and Rabbit Sera.

It may be remembered that studies of the paracolon group followed an investigation of outbreaks of gastro-enteritis in a R.A.A.F. camp, some cases being apparently due to these organisms. In order to determine the level of antibodies to paracolon bacilli, 31 sera were examined, these being grouped in the following manner: 14 from acute cases (obtained two weeks after the onset of symptoms), 3 from convalescent patients, 3 from asymptomatic food handlers in the camp and 11 from normal individuals. Nineteen paracolon strains, isolated from patients and normal individuals, were tested. Three kinds of broth cultures were used for agglutination tests: living, heated at 56° C. for 30 min. and boiled. As a result of agglutination reactions no correlation could be demonstrated between paracolon bacilli and antibodies formed in sera of patients and normal individuals with one significant exception in the case of paracolon *melbourne* strains, which will be discussed separately. Otherwise organisms isolated from stools of patients often gave negative results with sera of these patients whereas positive results were recorded with sera of other individuals. It was demonstrated that freshly

isolated strains often contained a variety of minor unstable antigens, lost on subculture; these fractions were responsible for frequent and irregular serological reactions, the agglutination being scanty with no distinct end point. Two of these unstable antigens were thermolabile at 52° C. Some sera displayed natural antibodies to a number of different antigens, especially contained in living cultures, while other sera gave constantly negative results.

TABLE 5.

Agglutination of normal human and rabbit sera against H, O and β suspensions of paracolon bacilli.

Antigens		Sera												
		Source	No.	P.c. non-agglutinating	P.c. agglutinating	P.c. agglutinating at titre								
Type	No.					1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560
H	8	Human	25	78	22	11	8	2	1	1	0	0	0	0
O	13			85	15	6	6	1	2	1	0	0	0	0
β	4			11	89	17	21	19	14	5	5	6	1	1
H	8	Rabbit	28	98	2	2	1	0	0	0	0	0	0	0
O	13			75	25	7	13	3	2	0	0	0	0	0
β	4			64	36	15	13	4	0	2	0	2	0	0

An additional investigation was made of 25 normal human and 28 normal rabbit sera to determine the level of natural antibodies to "stabilized" paracolon strains, which have lost their minor antigens in the course of frequent subculturing on artificial media. Twenty-five suspensions used for serological tests comprised eight H, thirteen O and four β suspensions. The level of natural antibodies as shown in Table 5 was higher in human than in rabbit sera, the titre in the former reaching 1/160 for H and O agglutinations, whereas in rabbit sera titre 1/20 was the highest observed for H and 1/80 for O reactions. It was noticed that the occurrence of β antibodies was frequent in human sera (89 p.c.), high titres, e.g. 1/2,560 being demonstrated. These results re-emphasize the importance of determining the level of antibodies against paracolon bacilli in the community before attempting to interpret the presence of antibodies against these organisms in cases of gastro-enteritis.

Variability in Cultural Characters, Biochemical Activities and Antigenic Structure of Paracolon Bacilli.

In the course of this study it became apparent that a number of paracolon strains exhibited variations in their cultural characters, biochemical reactions and antigenic structure. The description of a β paracolon strain, isolated from a case of gastro-enteritis, will illustrate these changes.

On primary isolation this organism showed on MacConkey medium smooth colonies with an entire edge. After two years' cultivation on agar slope under paraffin oil, plating out on MacConkey medium produced two types of colonies, viz. raised with an undulate edge (Fig. 1) and large, flat colonies, the latter becoming differentiated after additional incubation at 37° C. into a central pale mucoid part and a flat periphery covered with pink papillae (Fig. 2). Microscopic examination showed a marked pleomorphism of the bacilli, the mucoid part being composed of short thick rods and the papillae of long, thin ones. Agglutination tests revealed

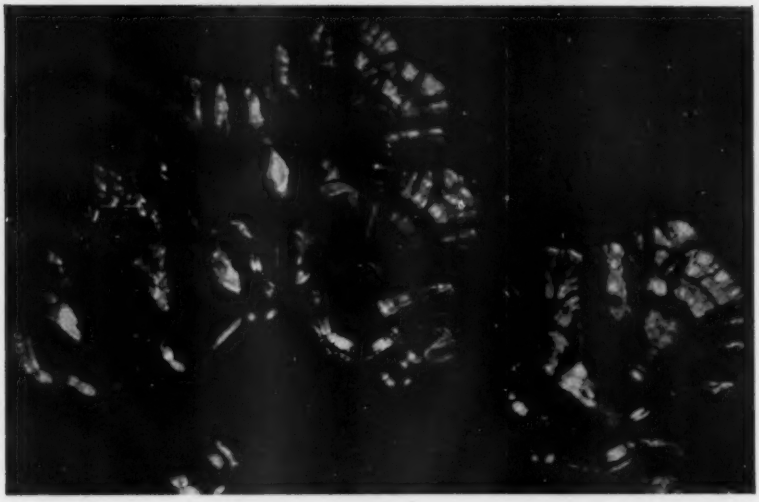

Fig. 1.

Fig. 2.

Figs. 1 and 2. Colony variants of a paracolon strain.

a variation in the antigenic structure of the mucoid part with the loss of β component while the papillae and the raised colonies retained their original structure. A change was induced also in the biochemical activities of this strain, the late lactose-fermenting anaerogenic paracolon becoming a gas producing coliform.

Variants with regard to saccharose, dulcite and salicin fermentation were demonstrated by the use of agar media, impregnated with the appropriate carbohydrate. The comparatively frequent occurrence of these variants prompted our decision to avoid biochemical classification based on the fermentation of saccharose and dulcite.

CLINICAL FINDINGS AND PATHOGENICITY TESTS.

An important problem of this investigation was the assessment of pathogenicity of various paracolon strains and their significance in gastro-enteritis. Two groups of paracolon bacilli were of special interest, the pathogenic strains and those with an ability to establish themselves in the intestinal tract without causing symptoms. In the first group we included paracolon *melbourne* strain. This organism appeared to have a definite causal relationship to an outbreak of acute gastro-enteritis in the R.A.A.F. camp. This outbreak, involving over 20 men, commenced with explosive suddenness, suggesting a common etiological agent from a common source. The symptoms were severe abdominal pains and diarrhoea with mucus and occasionally blood, the diarrhoea lasting approximately 8 hours. The incriminating vehicle seemed to have been custard eaten about 14 hours previously but unfortunately no remnants of this or other foods were available for bacteriological examination. Furthermore, for reasons outside our control, stools from only five of the cases and from one convalescent were subjected to bacteriological examination. In each case the same paracolon *melbourne* strain was found in predominant numbers. While admitting that the bacteriological work of this outbreak was not complete, the evidence strongly suggested that the causal agent was paracolon *melbourne* strain. This contention is supported by the results of serological investigation which showed an antibody response in sera of four out of five patients, the sera being obtained two weeks after the onset of symptoms. The titres were 1/20 to 1/40 with H antigen and 1/80 to 1/160 with O antigen. A comparative study with sera of eight patients suffering from gastro-enteritis due to other enteric pathogens, gave negative or 1/10 titre with H and no response with O antigen. A subsequent investigation of 25 normal sera showed in some cases titres 1/10 to 1/20 for H reactions but no antibodies were demonstrated with O antigen. As mentioned previously, paracolon *melbourne* strains formed a biochemically and antigenically homologous sub-group of *Paracolobactrum intermedium* type I.

Pathogenicity tests were carried out on human volunteers and laboratory animals. Five volunteers were fed with saline suspensions of paracolon *melbourne* harvested from growth on agar, the doses ranging from 100×10^6 to $5{,}000 \times 10^6$ bacilli. No clinical response was observed. However, this might have been due to two factors, viz. the culture was converting rapidly to a rough phase or possibly the suspensions from agar growth might have been devoid of originally present toxic substances. Pathogenicity tests on mice showed that fatal septicaemia was produced within 24 hours with 0·1 ml. of 18 hour broth cultures, injected intraperitoneally. Kittens fed with organs of mice, which succumbed to infection with paracolon *melbourne* strain, displayed severe symptoms of diarrhoea, the administered bacterium being recovered from faeces of the kittens in an almost pure culture.

On the basis of the above findings it is thought that sufficient evidence was produced for pathogenicity of paracolon *melbourne* strain.

Two other paracolon strains proved to be of interest owing to their ability of implantation in the bowel. Paracolon 32, belonging to *Paracolobactrum intermedium* type II, was

originally isolated from the faeces of a food handler with no symptoms of diarrhoea. Stools of this individual were examined eight times within six months and the same organism continued to form the predominant flora on MacConkey plates. The person harbouring paracolon 32 was given a two weeks' course of lactose but the same strain was recovered from his stools, whereas there was no difficulty in transforming this paracolon into a coliform bacillus *in vitro* in the presence of lactose.

Pathogenicity tests were performed on six volunteers who received *per os* $1{,}000 \times 10^6$ to $1{,}500 \times 10^6$ of paracolon 32 strain. The following day two of the six volunteers reported abdominal pains and loose motions with mucus. Prior to the feeding experiments the above paracolon bacilli were not detected in the stools of these individuals while after feeding this organism appeared in large numbers in the faecal specimens of five of the volunteers, including those displaying clinical symptoms. Antibodies in response to the administered organism were not observed in the sera of the volunteers. An occurrence worth recording took place in connection with one of the symptomless volunteers who reported a gastro-enteritic attack six weeks after the feeding experiment. A bacteriological examination of his stool revealed the presence of paracolon 32, which appeared on MacConkey plate in pure culture, thus indicating that the volunteer became a carrier of this strain.

Another interesting group of paracolon bacilli was recovered from stools of some inmates of the Mental Hospital who apparently were not involved at that time in any specific outbreak of gastro-enteritis. This group included six biochemically and antigenically identical strains which showed on isolation biochemical activities somewhat similar to *Salm. typhi* but their agglutination reactions with somatic antiserum of *Salm. typhi* gave only 1/32 of the full titre. The occurrence of these six identical strains in specimens from one institution was apparently due to cross-infection as shown by the significant fact that this strain was distinct from the other paracolon bacilli forming our collection of over 250 cultures.

Another aspect of clinical findings was the response to sulphaguanidine therapy, one or two courses of this drug being administered to some members of the R.A.A.F. personnel, from whose stools paracolon bacilli were cultivated. The outbreak of gastro-enteritis presumably caused by paracolon *melbourne* strain was not controlled from a bacteriological point of view by sulphaguanidine. The infection, however, was of short duration and subsided equally well with or without treatment. Paracolon 32, which has shown the ability of implantation in the bowel, was not detected two weeks after the administration of sulphaguanidine but re-appeared again in four weeks, thus giving evidence of temporary suppression. Another strain, paracolon 56, isolated in a pure culture from an acute case of gastro-enteritis was not affected by sulphaguanidine. On the other hand a number of cases seemed to have responded to therapy with this drug but it should be taken into consideration that usually only one bacteriological examination was made after the treatment which left the possibility of a suppressed organism re-appearing at a later date. The general picture was that the response to sulphaguanidine therapy was variable.

The in Vitro *Activity of Sulphaguanidine on Paracolon Bacilli and a Comparison with Clinical Findings.*

The *in vivo* treatment of paracolon bacilli with sulphaguanidine was followed up by *in vitro* estimation of sensitivity of these organisms to the drug. *Shigella* strains were included as means of comparison although it should be noted that *Shigella* were old stock cultures, while paracolon bacilli were isolated comparatively recently. The method recommended by Harper and Cawston (1945) was adopted. Concentration of the drug was expressed in mg. per 100 ml. of medium, 10 mg. being the lowest concentration tested.

TABLE 6.

Inhibitory action of sulphaguanidine on paracolon and Shigella *strains.*

Source of material	Type	No. tested	No. growing at mg. p.c. sulphaguanidine 100	75	50	40	30	20	10	No growth at 10 mg. p.c.
Abnormal	Paracolon sp.	20	7	2	0	1	4	3	3	0
Normal	Paracolon sp.	22	0	1	0	4	4	6	3	4
Abnormal	*Shigella*	15	1	0	0	0	4	2	3	5

Table 6 shows that there was no clear line of demarcation in susceptibility to sulphaguanidine of paracolon bacilli from normal and abnormal material. To compare the *in vitro* and *in vivo* results of the activity of sulphaguanidine, the six pathogenic strains of paracolon *melbourne* were least sensitive to the drug and were uninhibited at 100 mg. per cent. but the infection caused by these organisms was of too short duration to evaluate the efficiency of treatment. Paracolon 32 proved to be one of the most sensitive strains, its growth being inhibited at 10 mg. per cent., whereas *in vivo* it was only temporarily suppressed. Paracolon 56, isolated from a case of acute gastroenteritis, was not controlled by sulphaguanidine *in vivo*, although it was sensitive to this drug *in vitro*. Thus on the basis of the described experiments no correlation could be found between the results *in vivo* and *in vitro*.

DISCUSSION.

A review of literature shows that, when cases of gastro-enteritis could not be traced to known pathogens of *Salmonella* and *Shigella* groups, various other types of bacteria came under suspicion. One of the groups of organisms to attract attention were the paracolon bacilli. As mentioned previously, many conflicting views have been expressed with regard to their significance. Although reports are accumulating to show that some strains are responsible for acute enteric infections, in many cases the evidence is not fully convincing. At an early stage of these investigations it was recognized that paracolon bacilli form a heterogeneous collection of strains and various types were held responsible for infections, thus: *Bact. asiaticus* in cases of fever (Castellani, 1912), a group of haemolytic bacteria in acute infections of the urinary tract (Dudgeon, 1923), *Bact. coli-mutabile* strain in an outbreak of diarrhoea in the new-born (Dulaney and Michelson, 1935). Survey of recent literature gives additional evidence of pathogenic strains responsible for outbreaks of gastro-enteritis or associated with them. Rhodes (1942), Barnes and Cherry (1946), Christensen (1947), Plass (1947) reported outbreaks of diarrhoea in connection with paracolon bacilli. Sevitt (1945) described a group of paracolon strains, isolated from stools of children suffering with gastro-enteritis probably similar to Dudgeon's (1923) organisms cultured from patients with urinary tract infections. Ferris and Hertzberg (1945) gave an account of an epidemic of diarrhoea; the causative organism named by them *Salmonella atherton* fermented lactose after sev-

eral subcultures in that medium and was shown (in an appendix by Atkinson) to be serologically related to *Salm. arizona* which Kauffmann (1945) classified as belonging to *Salmonella-coli* group. Edwards *et al.* (1947) described the Arizona group of paracolon bacilli, fermenting lactose with varying avidity; they expressed little doubt with regard to the pathogenicity of these strains which were found in man, domestic animals and reptiles. The above writers (1948) published also a paper on the Bethesda group of paracolon bacilli, believing some members of this group capable of inciting gastro-enteritis, although no definite proof was procured.

As a result of our investigation it is claimed that evidence was obtained for pathogenicity of paracolon *melbourne*, this strain being the etiological agent of an outbreak of gastro-enteritis in a R.A.A.F. camp. Pathogenicity of this organism might have been increased by a favourable set of circumstances, e.g. production of enterotoxic substances in a custard medium (Jordan and Burrows, 1935). The statement of pathogenicity was based on the following facts: (a) the explosive suddenness of the outbreak involving simultaneously a number of people; (b) similarity in clinical manifestations of the illness; (c) isolation of biochemically and antigenically identical bacilli from stools of the examined patients; (d) antibody response in the sera of these individuals; (e) virulence of this strain to mice by intraperitoneal injections and to kittens by feeding.

Paracolon *melbourne* strain differs biochemically from members of the Arizona group and its flagellar antigen does not resemble factors z_4, z_{23}, z_{24} and g appearing in that group. A comparison with Bethesda strains shows a likeness in biochemical activities. A serological dissimilarity was demonstrated between paracolon *melbourne* and a number of paracolon bacilli which biochemically resembled Bethesda strains and which we isolated in the course of our studies from faecal specimens of normal individuals as well as from gastro-enteritic cases.

Edwards *et al.* (1948) pointed out in their study on the Bethesda strains that the question of pathogenicity of this group was not answered. Similarly, Stuart *et al.* (1948) did not supply complete data on the pathogenicity of *Paracolobactrum intermedium* cultures described by them as resembling Bethesda. Therefore, we suggest to regard members of Bethesda group as potentially pathogenic, labelling only those which were proved to be incriminating factors in outbreaks of gastro-enteritis, as in the case of paracolon *melbourne*.

On the basis of feeding experiments with human volunteers it seems possible that, following the administration of large doses, some strains of paracolon bacilli may produce a mild infection or a symptomless carrier state. This was the case with paracolon 32. Further, isolation in one institution of six identical strains of an uncommon type of paracolon indicated some degree of invasiveness, probably due to predisposing factors (Stuart *et al.*, 1943).

Consideration of statistical data in literature on the occurrence of paracolon bacilli in stools shows a wide range of variation (Sandiford, 1935; Kriebel, 1936; Dulaney and Smith, 1939; Stuart *et al.*, 1940; Sevitt, 1945). However, the general picture of these findings as well as our results indicate a higher ratio and a higher count of these organisms in stools from cases of gastro-enteritis over that from normal individuals. Apart from the problem of pathogenicity the question as to whether these slow lactose-fermenters are variants of recognised pathogens (viz. *Salmonella* and *Shigella*) or variants of coliform bacilli with a retarded ability to attack lactose is as yet unsettled. A third alternative is that the altered flora indicates a response to changed conditions of the intestine, favouring the rapid multiplication of normally scanty paracolon bacilli. In isolated cases evidence was brought forward for transformation of some pathogens, e.g. *S. flexneri* (Smith, 1941), *S. sonnei* (Stuart *et al.*, 1940) into coliform bacilli. Saphra and Seligman (1947) in reporting a lactose fermenter with the antigenic formula of *Salm. newington* could not decide whether the change progressed from *Salmonella* to *Escherichia coli* or vice-versa. Kriebel (1936) observed dissociation of coliform cultures into late lactose fermenters.

Our studies demonstrated a continuity in serological relationship between some *Shigella*, paracolon and coliform strains, this affinity being due to the presence of a common β antigen which was encountered in strains from normal as well as abnormal material. We have found it possible to convert a number of very late lactose-fermenters into coliform bacilli but no proof was obtained *in vivo* for such a transformation. With regard to the increase of paracolon flora in stools of gastro-enteritic and convalescent patients our results point in some cases to the pathogenic or potentially pathogenic paracolon strains as etiological agents and, in other cases, to paracolon bacilli serving as indicators of an intestinal injury.

An important problem is the classification of paracolon bacilli. From the point of view of taxonomy classification based on biochemical reactions serves a useful purpose. Consideration must be given, however, to the presence of variable forms, occurrence of "shifts" (Parr, 1939) being a noticeable feature. In our experience IMViC reactions gave more stable means of differentiation than fermentation of various carbohydrates, viz. sucrose and dulcite. We noticed also, that stock cultures gave more consistent results than freshly isolated ones. Our work and the work of others (e.g. Stuart *et al.*, 1943) shows that there is no constant correlation between a biochemical and serological pattern.

Many attempts have been made with regard to serological classification of paracolon bacilli. At first this proved futile (Kriebel, 1936). In 1947 Kauffmann described a serological diagnostic schema for the coliform group, which included some late lactose fermenters; in this classification O antigens played the main part, including new antigens L, A and B grouped together under the symbol K. Further studies showed that bacilli belonging to these groups may contain other somatic antigens, e.g. α (Stamp and Stone, 1944), β (Mushin, 1949). Edwards *et al.* (1943) did not consider it practical to introduce a serological diagnostic schema for all paracolon bacilli but advocated the study of groups of organisms which were suspected of being etiological agents of infection. To demonstrate the complicated structure of these organisms it may be mentioned that Arizona group comprising 456 cultures, was divided by Edwards *et al.* (1947) into 19 somatic groups and 55 types.

Our findings have shown that serological cross-reactions existed between paracolon strains from normal and abnormal material and also between these organisms and *Salmonella* and *Shigella*. A survey of literature (Stuart *et al.*, 1943; Kauffmann, 1944) also brought under notice the presence of common antigens in paracolon and coliform bacilli on one side and *Salmonella* and *Shigella* on the other side. Taxonomically this is of interest; however, the sharing of an antigen between a paracolon and a recognized pathogen does not implicate virulence. Besides, common antigens have been found amongst such diverse types as *Bact. aerogenes*, Pneumococcus type II and Friedländer's bacillus (Julianelle, 1937).

It seems to us that considering the records of complexity of antigenic structure of paracolon bacilli, their serological classification should be limited to strains of proved pathogenicity until more practical criteria of differentiation can be established.

SUMMARY.

Survey of frequency of paracolon bacilli in stools from gastro-enteritic cases and from normal individuals showed a higher ratio in the abnormal material. Plain desoxycholate agar proved to be the most selective medium for the isolation of these organisms.

Biochemical and serological investigations showed affinity between paracolon strains from pathological and normal specimens and cross-agglutinations with some *Salmonella* and *Shigella* antisera.

Natural antibodies to some paracolon strains were demonstrated in normal human and rabbit sera, especially to unstable antigens contained in freshly isolated cultures and to β antigen.

Variability was studied with regard to morphology, biochemical reactions and antigenic structure of paracolon bacilli.

Paracolon *melbourne* strain was proved to be the etiological agent in an outbreak of gastro-enteritis.

Two paracolon strains showed an ability of implantation in the intestinal tract without causing symptoms. One of these strains administered to human volunteers *per os* in large doses induced mild gastro-enteritis or a carrier state.

Estimation of sensitivity of paracolon bacilli to sulphaguanidine demonstrated lack of correlation between the *in vivo* and *in vitro* results.

The results of our studies on the rôle of paracolon bacilli in intestinal flora point to some strains as etiological agents of gastro-enteritis and to a significant increase of other strains as an indication of metabolic disorders.

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INHIBITION OF INFLUENZA VIRUS HAEMAGGLUTINATION BY MUCOIDS

II. DIFFERENTIAL BEHAVIOUR OF MUCOID INHIBITORS WITH INDICATOR VIRUSES

by JOYCE D. STONE[1]

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(Accepted for publication 18th July, 1949.)

In an earlier paper (Stone, 1949) techniques were described for converting a number of strains of influenza virus to the "indicator" state in which their haemagglutinating action is highly sensitive to inhibition by certain mucoids. The present work is concerned with the use of these indicator viruses in studying the nature of inhibitors.

The kinetics of the enzymic inactivation of inhibitor by influenza virus as revealed with the standard indicator, heated LEE influenza B virus, have been studied by Burnet (1948). However, with the variety of indicators now available it has been possible to obtain further information about the inhibitors and their enzymic inactivation. In the present work it has been shown that the inhibitory activity of mucoid for the different indicator viruses is destroyed by virus action, or by the action of the cholera enzyme (RDE), in a certain definite order according to a "gradient" in a similar way to the agglutinability of red cells (Burnet, McCrea and Stone, 1946). The order in which the indicators fall in the gradient is characteristic of a particular inhibitor but varies with different mucoids.

Materials and Methods.

Viruses. The following strains were used in the form of infected allantoic fluid harvested from eggs inoculated at the age of 11 days and incubated for 48 hours:

Influenza A—egg-adapted substrain of WS (WSE), PR8 and Melbourne (MEL);
Influenza B—LEE.

Indicator viruses (Stone, 1949), denoted by the prefix H=(heated) or T=(treated), were prepared as follows:

H–WSE—allantoic fluid heated at 52° C. for 30 minutes;
T–PR8 and T–MEL—allantoic fluid diluted 1:5 in citrate-borate-saline and heated at 56° C. for 30 minutes;
H–LEE—allantoic fluid heated at 56° C. for 30 minutes.

Normal Saline contained 0·9 p.c. sodium chloride in distilled water.

[1] This work was carried out under a grant from the National Health and Medical Research Council, Canberra, Australia.

Citrate–Borate–Saline contained 10 p.c. by volume of borate buffer (prepared according to Palitzsch, see Clark, 1920) at pH 8·5 and 0·2 p.c. sodium citrate in normal saline.

Calcium–Acetate–Saline. Isotonic sodium acetate-acetic acid buffer adjusted to pH 6·2 and containing 0·1 p.c. calcium chloride.

Ovomucin supplied through the courtesy of Dr. A. Gottschalk, was prepared as described in Gottschalk and Lind (1949a). The titres refer to a stock solution containing 0·2 p.c. (dry weight) in normal saline.

Cyst Mucoid purified from the contents of an ovarian cyst by the method of McCrea (1949) was kindly prepared by Mr. E. L. French. The stock solution contained 1 p.c. of dried material in normal saline.

Sheep Salivary Mucin was kindly supplied by Dr. J. F. McCrea. It had been prepared from submandibular glands according to his method to be published. Titres refer to a stock solution containing 1 p.c. (dry weight) in normal saline.

Receptor-Destroying Enzyme (RDE) of V. cholerae. Agar extract from cultures of the 4Z strain was prepared by the method of Burnet and Stone. Titres of RDE were determined as previously described (Burnet and Stone, 1947).

Titration of Inhibitor. Serial twofold dilutions of inhibitor were prepared in 0·25 ml. of normal saline. An equal volume of a dilution containing five agglutinating doses of the appropriate indicator virus was added to each tube and the mixtures allowed to stand for 30 minutes at room temperature (20–24° C.). A third volume (0·25 ml.) of a 1 p.c. suspension of inhibitor-sensitive fowl cells (Anderson, 1948) was added and the test read by the pattern of haemagglutination after a further hour at room temperature. The end-point was the "+" degree of partial haemagglutination.

EXPERIMENTS.

The "Indicator Gradient" of Various Mucoids.

In studying the enzymic action of virus or RDE on an inhibitor the general technique consisted in incubating an appropriate dilution of inhibitor (approximately 1,000 inhibitory doses for H–LEE) in calcium-acetate saline with enzyme at 37° C. Virus was used in concentrations ranging from 1 to 10 agglutinating doses. Samples were removed at intervals and, after heating under conditions which destroyed the enzyme without affecting the inhibitor (1 minute at 100° C. for cyst mucoid and sheep mucin, 30 minutes at 65° C. for ovomucin), these were titrated for inhibitory activity against a series of indicator viruses. A control tube of mucoid was incubated without enzyme and samples, taken at the beginning and end of the experiment, heated in the same way as the test samples. In expressing the results in the Figures the inhibitory activity for each indicator has been shown as the percentage of the control value for the corresponding indicator.

(1) Action of Virus on Ovomucin.

Fig. 1 shows the results obtained when ovomucin was treated with MEL influenza A virus and LEE influenza B virus. It will be seen that the extent of the reduction in inhibitory titre which could be demonstrated depended on the indicator virus used to titrate the samples. In the results with LEE enzyme there was no significant change in inhibitor as tested with T–MEL but when H–WSE or H–LEE was used as indicator the inhibitory titre showed marked reduction. When MEL virus was used as enzyme there was a sharp decrease in the inhibitory titre for T–MEL as well as for the other two indicators.

Fig. 2 shows three comparable experiments in which the viruses, MEL, LEE and WSE were used as enzymes and the inhibitory titre determined with indicator forms of these viruses. In each case the relative order of the curves with the three indicators was the same, H–WSE, H–LEE, T–MEL.

Thus it appeared that the indicator viruses could be arranged in a gradient according to the order in which the inhibitory activity for each was destroyed by virus action. The corresponding virus enzymes varied in the extent to which they destroyed the inhibitory titre for all indicators but, again, they fell in the same order as found for the indicator gradient. However small the effect as tested by heterologous indicators, each virus exerted a well marked action on the inhibitory titre as assayed with its own indicator phase.

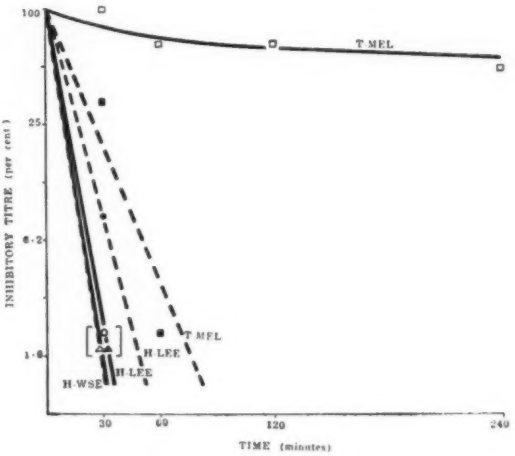

Fig. 1. Action of virus on ovomucin.
———— Enzyme—LEE
– – – – – – – – Enzyme—MEL

Each sample was titrated with three indicator viruses as shown on curves. The points in brackets represent titres less than the values shown.

In order to make a more detailed analysis of the kinetics of virus action on ovomucin the experiment shown in Fig. 3 was carried out using two concentrations of MEL virus (1 and 2 agglutinating doses) as enzyme and the indicators, H–LEE, H–WSE and T–MEL for titrating

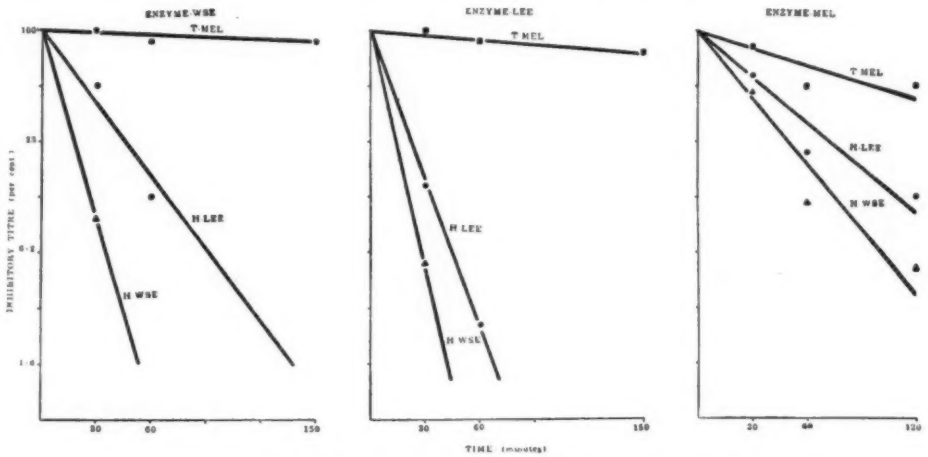

Fig. 2. Action of WSE, LEE and MEL viruses on ovomucin. Each sample was titrated with three indicators.

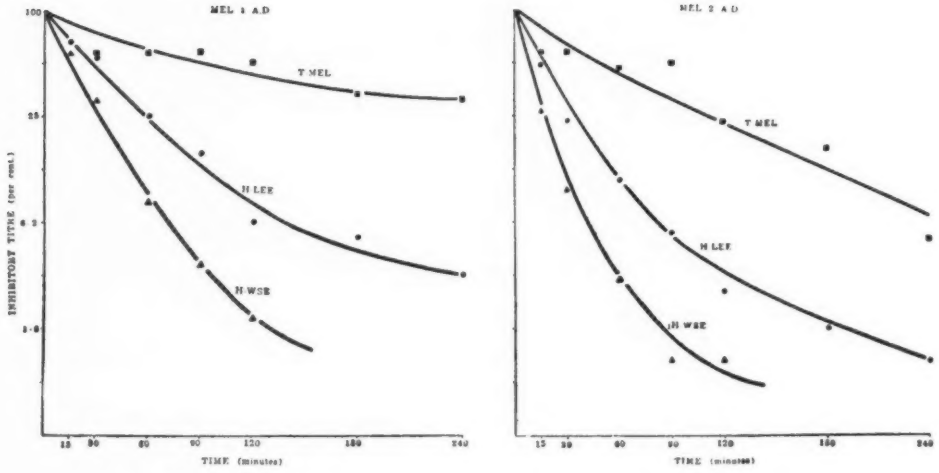

Fig. 3. Kinetics of inhibitor-destruction by MEL virus. Each sample of ovomucin was titrated with three indicators.

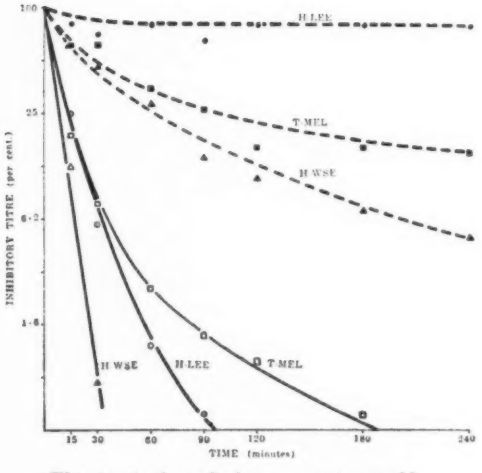

Fig. 4. Action of virus on cyst mucoid.

——— Enzyme—LEE
– – – – – – – Enzyme—MEL

Each sample was titrated with three indicators.

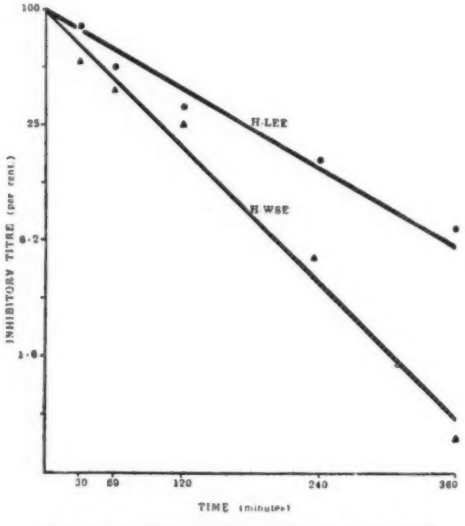

Fig. 5. Action of LEE virus on sheep salivary mucin. Each sample was titrated with two indicators.

agglutinating doses) as enzyme and the indicators, H–LEE, H–WSE and T–MEL for titrating inhibitory activity. The curves show the general shape found in all experiments using virus as enzyme: the reduction in inhibitor is logarithmic at first and then slackens, approaching finally a constant level.

(2) Action of Virus on Cyst Mucoid.

Fig. 4 shows the results of an experiment in which cyst mucoid was treated with MEL and LEE viruses. Considering first the results with MEL enzyme it will be seen that the indicator viruses give a different gradient from that found with ovomucin. H–WSE is again the most sensitive indicator of enzyme action, but the order of the other two is reversed, in

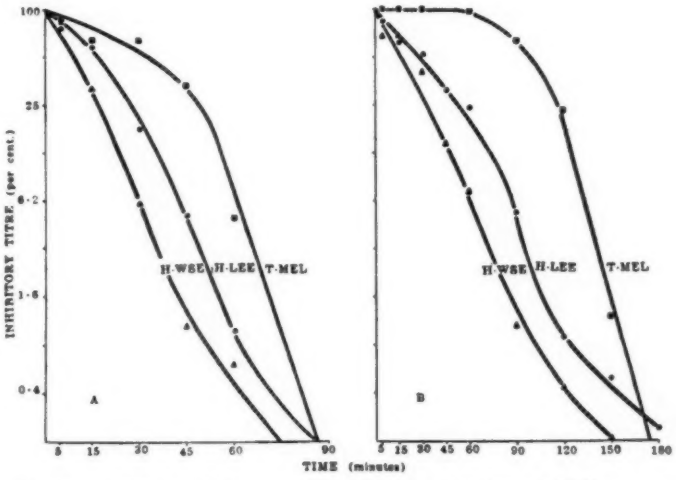

Fig. 6. Action of RDE on ovomucin. Concentration of RDE:
A. 7 units. B. 3·5 units.
Each sample was titrated with three indicators.

fact with H–LEE there was no significant reduction in inhibitory titre over the test period of four hours. When LEE virus was used as enzyme the titres with H–WSE were again reduced most readily. The curves with H–LEE and T–MEL fell very close together in the early part of the reaction but later the inhibitory titre for H–LEE showed greater reduction than that for T–MEL. Comparing the results with the two virus enzymes it will be seen that, in confirmation of Burnet's finding (Burnet, 1948), LEE had a more extensive action on the inhibitor than did MEL. This was evident both from the slope of the logarithmic part of the curve and the height of the asymptote towards which the curve tended.

(3) Action of Virus on Sheep Salivary Mucin.

Anderson (1948, unpublished) has shown that extracts of sheep salivary glands contain an inhibitor for heated LEE virus and that the inhibitor is inactivated by RDE from *V. cholerae*.

In preliminary tests of the action of virus on sheep mucin variable results were obtained

and it appeared that the different batches of mucin were not uniformly susceptible. However, using certain preparations it was possible to demonstrate inactivation of the inhibitor with the technique described above.

The effect of LEE virus on a solution of semipurified sheep mucin is shown in Fig. 5. It will be seen that the inhibitory activity for H–WSE was more readily destroyed than that for H–LEE. It was not possible to use T–MEL in the experiment as this indicator is not significantly inhibited by sheep mucin.

(4) Action of RDE on Ovomucin.

In order to determine whether there was a gradation, similar to that shown with virus, in the action of RDE on the inhibitory activity of soluble inhibitor for the different indicator viruses, experiments of the type described above were made with RDE and ovomucin.

As will be seen from Fig. 6 ovomucin was modified by the action of RDE so that it lost its inhibitory activity for H–WSE most readily, then for H–LEE and T–MEL. The gradient therefore corresponds with that given by virus action. However, the shape of the curves obtained was quite different. From a comparison of Figs. 3 and 6 it is evident that the curves with RDE are at first divergent but then tend to come together in the later part of the reaction, whereas those obtained with virus diverge at the beginning and either remain divergent or become approximately parallel. This general picture of the curves with the two reagents was constant in a number of experiments made.

Relationship of Inhibitors Assayed with Different Indicators.

Absorption with PVR-Cells.

It has been shown by Fazekas de St.Groth and Gottschalk (1949) that the inhibitor in cyst mucin or ovomucin can be removed by physical adsorption to virus-coated red cells. To determine whether it could be shown that the inhibitory activity for the various indicators is associated with different fractions, ovomucin was absorbed with virus-coated red cells and the residue titrated for inhibitor with a series of indicators. "PVR" cells (Fazekas de St.Groth, 1949) which had been coated with virus by successive treatments with periodate, virus and RDE were used for absorption carried out at 0° C. as described by Fazekas de St.Groth and Gottschalk, 1949.

TABLE 1.

Absorption of ovomucin with LEE-coated (PVR) cells.

Number of absorptions	Inhibitory titre*			
	H–WSE	H–LEE	T–MEL	T–PR8
1	<0·2	<0·2	8	24
2	<0·2	<0·2	8	22
3	<0·2	<0·2	4	12
4	<0·2	<0·2	4	9

* Expressed as percentage of original titre for the same indicator.

Table 1 shows the results obtained when ovomucin was absorbed with PVR cells coated with LEE virus. Titres with the different indicator viruses are expressed as the percentage of the titre given with the original solution of ovomucin. It will be seen that a single absorp-

tion with LEE-coated cells removed the inhibitory activity for H–LEE and H–WSE and caused a marked reduction in the titres against T–MEL and T–PR8. Further treatment reduced the titres for T–MEL and T–PR8 a little further but even with four successive absorptions considerable activity against these indicators remained.

Solubility at Various pH Levels.

In an experiment carried out in collaboration with Mr. G. L. Ada the solubility of ovomucin was tested in buffers of various pH levels in the range 3·8 to 9·5. The solutions were titrated for inhibitory activity against the indicator viruses, H–WSE, T–MEL and H–LEE, in order to see whether there was any evidence of differentiation of inhibitors by their solubilities.

A concentrated preparation of ovomucin (1 p.c. in 5 p.c. saline) was mixed with two volumes of buffer and held for a period of three days at room temperature with occasional shaking. The mixtures were then centrifuged at low speed and the supernatant fluids titrated for inhibitory activity, making a preliminary 1:100 dilution in saline buffered with phosphate to pH 7·0.

TABLE 2.

Inhibitory activity of ovomucin solutions prepared at different pH levels.

Buffer		Inhibitory titre*		
Composition	pH	H–WSE	H–LEE	T–MEL
Acetate	3·8	34	50	41
Acetate	4·8	73	67	68
Phosphate	6·2	73	90	82
Phosphate	7·0	100	100	100
Phosphate	7·8	135	117	109
Borate	8·6	135	117	109
Borate	9·5	123	117	109

* Expressed as percentage of "phosphate pH 7·0" values.

In Table 2 the results are expressed as the percentage of the titre given by the solution in buffer at pH 7·0 with the corresponding indicator. There was evidence of decreased solubility at the more acid pH levels but as the lower titres were obtained with all indicators there was no suggestion that the inhibitory activity for the three indicators was dependent on fractions with different solubilities.

Inhibitory Spectrum of Mucoids with Different Indicators.

Table 3 shows the inhibitory titres of cyst mucoid, ovomucin and sheep mucin with the indicators H–WSE, H–LEE and T–MEL. It will be seen that the ratio of the titres given with the different indicators varied according to the inhibitor used. Although there was some variation in the exact ratios obtained with different batches of mucoid and of indicator virus, the general pattern obtained was characteristic of any inhibitor. In all experiments the inhibitory titres of cyst mucoid were slightly higher using H–LEE than the other indicators, whereas in the case of ovomucin T–MEL gave titres 3 to 6 times higher than H–LEE. Sheep mucin failed to inhibit T–MEL although it was active against H–LEE and H–WSE.

TABLE 3.

Inhibitory spectrum of mucoids with different indicators.

Inhibitor	Inhibitory titre*		
	H–WSE	H–LEE	T–MEL
Ovomucin	3,000	3,000	18,000
Cyst mucoid	20,000	24,000	12,000
Sheep mucin	28,000	7,000	<100

* Titres refer to the stock solutions of the different mucoids (see Methods).

DISCUSSION.

A close parallel between the enzymic action of influenza viruses on soluble mucoid inhibitors and on the virus receptors of the red cell is revealed in the results reported in this paper. When the course of the destruction of inhibitor was followed with several different indicator viruses it was found that the reaction was a complex process in which separate stages could be differentiated biologically by the disappearance of inhibitory activity for the individual indicator viruses. This appeared to be analogous to the phenomenon of red-cell receptor destruction by virus where the agglutinability of the red cell for different viruses is destroyed in a certain definite order, according to a "receptor gradient" (Burnet, McCrea and Stone, 1946). In the same way, from the results obtained in the inactivation of an inhibitor, the indicator viruses can be arranged to form an "inhibitor gradient" which is characteristic of that particular inhibitor.

In its reaction with the red cell each virus can destroy the agglutinability of the cell for all viruses preceding it in the receptor gradient but not for those falling after it. Similarly the capacity of a virus to destroy the inhibitory activity of a mucoid for the different indicators corresponds with the position of its indicator form in the "inhibitor gradient". For example, in the gradient for ovomucin, H-WSE and H-LEE appeared to be clearly separated from T-MEL and, in their enzymically active form, WSE and LEE readily destroyed the inhibitory activity of ovomucin for H-WSE and H-LEE but not that for T-MEL. MEL enzyme, on the other hand, removed the inhibitory titre against all three indicators.

On the basis of these results, MEL virus can be regarded as exerting a more extensive action on the biological activity of ovomucin than either WSE or LEE. This conclusion has received support at the chemical level by the finding of Gottschalk and Lind (1949 b) that MEL virus produces a larger amount of dialysable split product than LEE when it reacts with ovomucin. Prelimin-

ary tests of inhibitor destruction by other members of our standard series of viruses would suggest that mumps resembles LEE and WSE in being unable to destroy the inhibitory activity of ovomucin for T-MEL while NDV, PR8, SW and BON, like MEL, exert a more extensive action (Stone, 1949).

The correspondence between the position of the indicator form of a virus in the inhibitor gradient with the extent to which the virus can modify the inhibitor would suggest that the behaviour of the indicator form is of considerable significance. Both reactions are presumably governed by the adsorptive affinity of the virus for the inhibitor. If, as has been suggested (Stone, 1949), the affinity for the inhibitor is increased in the change to indicator, it must be that the relative order of the viruses, i.e. the gradient, is retained during this modification.

With ovomucin the order in which the inhibitory activity for the indicators H-WSE, H-LEE and T-MEL was reduced by the corresponding virus enzymes was the same in each case. When cyst mucoid was used, however, the order for MEL enzyme was H-WSE, T-MEL, H-LEE, but for LEE enzyme the curves obtained with the three indicators fell in the order H-WSE, H-LEE, T-MEL, although those with H-LEE and T-MEL were practically superimposed in the early stages. Similar discrepancies in enzyme action were noted by Burnet (1949) in a study of the final level to which the inhibitory titre of mucoid is reduced by virus action. In several instances the destruction of inhibitory power was more complete when assayed with the homologous indicator, than would be expected from the titres obtained with the other indicators in the gradient. These findings would suggest that, in addition to the action governed by its position in the gradient, a virus might cause further specific destruction in inhibitory titre for its own indicator phase.

The inter-relationship of the inhibitor-destruction curves as assayed with various indicators is characteristically different for the virus enzyme and for RDE of *V. cholerae*. In the case of virus the curves remained either divergent or parallel but with RDE they became convergent in the later part of the reaction. This points to a fundamental difference between the behaviour of the two enzymes which is probably associated with their nature, whether molecular or carried on the relatively large virus particle.

In considering the subject of inhibitory activity against different indicators, the question which immediately arises is whether a single chemical substance is concerned or whether materially distinct inhibitors can be differentiated. The biological results of the enzymic inactivation of inhibitor are capable of interpretation on either of two hypotheses. On the first one would assume that the inhibitory activity for the various indicators is associated with a single substance in which successive changes result in loss of activity against the indicator viruses in a definite order according to their positions in the gradient, and which

can be modified to various extents by the different virus enzymes. Secondly, there exists the possibility that a number of related substances is present, all of which serve as inhibitor for an indicator virus at the later end of the gradient, or as substrate for the corresponding enzyme, while only some can react with a virus early in the gradient (WSE in ovomucin).

A means of testing these hypotheses is provided by the absorption technique with virus-coated red cells (Fazekas de St.Groth and Gottschalk, 1949). If the second one held it should be possible to obtain fractions which differed in the pattern of inhibitory titres for various indicators according to the virus used in the absorption. Also, one might expect that similar fractions could be differentiated by chemical means.

In the present work there was no evidence that the inhibitory activity of ovomucin was associated with fractions of different isoelectric points. In experiments on the absorption of inhibitor from ovomucin with virus-coated red cells it appeared that there was preferential absorption of the inhibitory activity for H-WSE and H-LEE as compared with T-MEL. The inhibitory activity for T-MEL was considerably reduced by absorption with LEE-coated cells but not completely removed even after repeated absorption. It has been found by Fazekas de St.Groth and Gottschalk (1949) that cells coated with MEL removed all inhibitory activity against T-MEL in addition to that for H-LEE. The experimental findings are therefore in accordance with the second hypothesis in which the inhibitor may be considered as containing a population of units of very similar chemical properties but which show a range of adsorptive patterns varying from one which enables adsorption of all indicator viruses to one which is adequate for a single virus only. It is this pattern in relation to the indicator viruses which determines the "gradient" for any given indicator.

SUMMARY.

The mucoid inhibitors, ovomucin, cyst mucoid and sheep salivary mucin, were treated with influenza virus and the inhibitory titres assayed with three different "indicator" viruses. The mucoid lost its inhibitory activity for the various indicators in a certain definite order so that the indicator viruses could be arranged in a gradient corresponding with the receptor gradient obtained in the action of virus on the red cell. The gradient was characteristic of a particular mucoid but varied from one mucoid to another.

The strains of virus differed in their capacity to destroy the inhibitory titre of mucoid for the different indicators.

The same gradient was obtained in the action of the cholera enzyme (RDE) on ovomucin as in virus action.

Absorption of ovomucin with virus-coated cells removed the inhibitor as assayed with homologous indicator. Its effect on the inhibitory titre for heterologous indicator varied according to the position in the gradient of the virus used in the absorption.

Acknowledgment. It is a pleasure to thank Professor F. M. Burnet for his interest and criticism.

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PHOTOCHEMICAL DEGRADATION OF CELLULOSE IN THE PRESENCE OF TUNGSTIC ACID

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The oxides or acids of hexavalent tungsten and molybdenum, two neighbouring elements in group VIA of the periodic system, when finely dispersed on dry cellulose or other polysaccharides or certain of their derivatives turn blue if exposed to light of wavelength between 230 and 390 mμ. (Bolliger, 1946; Bolliger and Hinks, 1948). Thus, filter paper or materials consisting of cotton or artificial silk moistened with tungstic or molybdic acid and then dried become blue after a short period of exposure to the wavelengths mentioned above. This reaction in the practically solid state is confined to certain types of carbohydrates and, for example, does not take place with proteins, as demonstrated by the following experiment. If a woven material consisting of cotton and wool or natural and artificial silk threads is treated with tungstic or molybdic acid and irradiated with ultraviolet light, only the cellulose or artificial silk threads turn blue, while the threads consisting of proteins remain unaffected.

It may be assumed that the formation of the blue colour is due to a reduction of the colourless hexavalent tungsten and molybdenum to the blue oxides of lower valency. Light is necessary for this part of the reaction.

In order to establish the effective wavelengths, a tungstic or molybdic acid preparation on filter paper was placed in the plate-holder of a quartz spectrograph with a mercury arc as light source. Specific regions of the spectrum then registered themselves on the paper. In the visible region the preparations turned blue only at wavelengths less than 390 mμ. Furthermore blue colour lines of varying intensity could be seen throughout the near ultraviolet down to 230 mμ. Thus, only light in the near ultraviolet is effective in causing the colour change. Moreover it appears that the spectrum recorded on these papers is essentially identical with that obtained on photographic plates using the same light source.

In the case of molybdic acid the blue colour produced by light is retained permanently, but with tungstic acid the colouration is slowly lost in air if light of less than 390 mμ. is excluded. Increase of atmospheric humidity accelerates the rate at which the blue colour disappears and moistening of the blue preparation with water abolishes the colour rapidly. On re-exposure to light the blue colour is restored after the preparation has become air dry. The reaction of colour disappearance of tungsten preparations may be considered to be a re-oxidation of the pentoxide, W_2O_5, to the trioxide, WO_3. This process, however, takes place

only in the presence of moisture. Blue tungsten preparations kept in the dark in a dessicator containing phosphorus pentoxide or other dessicants retain their colour indefinitely.

This suggests that the oxygen required for the re-oxidation of the W_2O_5 is derived from water such as the moisture of the atmosphere.

Certain polysaccharides are required for the photochemical development of the blue colour and of these cellulose was examined for degradation products. Filter papers impregnated with tungstic or molybdic acid were exposed to the rays of a mercury arc or to sunlight behind a plateglass window. Both types of preparation turned rapidly blue from such irradiation. If subsequently protected from ultraviolet light the tungstic acid papers lost their colour; the molybdic acid preparations, however, retained their colour permanently.

Effects of Sunlight.

Considering firstly the effects of sunlight, tungsten and molybdenum preparations on filter paper turned blue during the day. During the night only the tungsten papers lost their colour, this being retored again by the morning sun.

After every second or fourth day a tungstic and a molybdic acid paper were analysed for: (a) Water soluble reducing compounds and (b) Total reducing compounds.

The results are summarized in Fig. 1, where the percentages of reducing substance expressed as glucose are correlated with the time of exposure to sunlight. Over a period of 13 days' exposure to sunlight there was no increase of water soluble reducing substances of the molybdic acid papers. The amount of total reducing substances was not significantly different from controls treated only with a comparable amount of sulphuric acid (0·05N). The tungstic acid preparations, however, showed a tenfold increase in total reducing substances compared with the molybdic acid papers. In contrast to the practically unaffected molybdenum papers the water soluble reducing substances amounted to 0·5 p.c. in the tungsten paper. Longer exposure to sunlight did not materially increase the reducing substances of these tungsten preparations as shown by the fact that after 50 days' exposure the total reducing power of the tungsten preparations was equivalent to 2·6 p.c. glucose, whereas, after 10 days the corresponding value was 2·2 p.c.

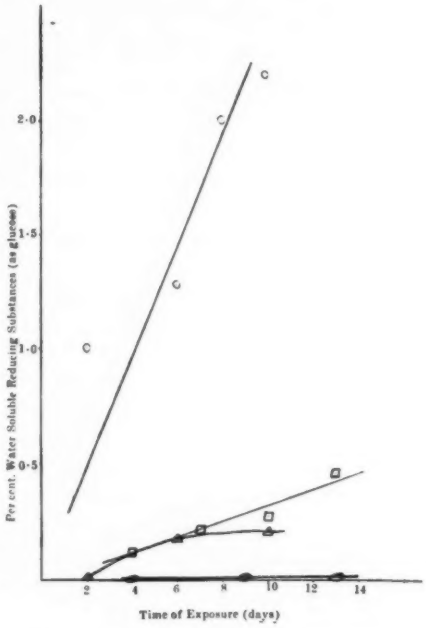

Fig. 1. Reducing substances produced on tungsten and molybdenum preparations (Filter paper Whatman No. 44 moistened with an approximately 1 p.c. solution of tungstic or molybdic acid and then dried), exposed to sunlight.

- O Tungsten preparation total reducing substances.
- □ Tungsten preparation water soluble reducing substances.
- △ Molybdenum preparation total reducing substances.
- ⬭ Molybdenum preparation water soluble reducing substances.

After 10 or more days' exposure to sunlight the deep blue molybdenum papers still retained their original tensile strength and texture, while the tungsten papers became brittle and showed a great tendency to break and tear. Simultaneously the colour responses diminished in intensity, that is to say, the blue colour expected from exposure to sunlight was slowly replaced by a permanently yellow tinge suggesting that a non responsive tungstic oxide had been formed. However, on moistening the papers with fresh tungstic acid the blue colour re-formed to some extent on exposure to suitable radiation. The content of reducing substances then increased further.

Intermittent Irradiation with an Artificial Light Source.

Filter papers treated with tungstic acid were irradiated with a mercury arc (Hannau, Hoehensonne), the distance between the arc and the papers being 45 cm.

It was repeatedly found that irradiation for 1 hour or less with this light source produced negligible amounts of water soluble reducing substances. The effect of interrupting the irradiation so as to apply radiant energy intermittently was then investigated, this intermittency being somewhat comparable with the alternations of day and night of solar irradiation.

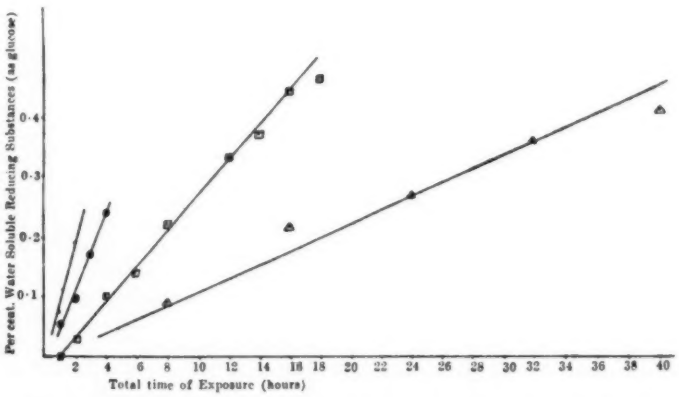
Per cent. Water Soluble Reducing Substances (as glucose)
0·4
0·3
0·2
0·1
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Total time of Exposure (hours)

Fig. 2. Water soluble reducing substances produced on tungsten preparations (Filter papers Whatman No. 44 uniformly moistened with a 2 p.c. tungstic acid solution and then dried) by intermittent radiation with a mercury arc.

- • 5 min. exposures.
- O 15 min. exposures.
- □ 60 min. exposures.
- △ 480 min. exposures.

The periods of irradiation in individual experiments were multiples of 5, 15, 60 and 480 minutes. After each irradiation the paper was placed in an atmosphere saturated with water at 30° C. and was kept there till the blue colour had fully disappeared. After 5 minutes of irradiation this took about 90 minutes and after 480 minutes of irradiation about 12 hours. The paper was then re-exposed to the rays of the mercury arc and this sequence of irradiation

followed by a rest period repeated till the total period of irradiation was completed. A part of the paper was then analysed for water soluble reducing substances as described previously (Bolliger and Hinks, 1948). The findings are summarized in Fig. 2. It is evident from the results obtained from multiple intermittent irradiation that the amount of water soluble reducing substances increases proportionately with the number of exposures forming an approximately straight line on the graph. The time of the individual exposure, however, is of considerable significance inasmuch as the amount of water soluble reducing substances formed by a number of intermittent irradiations of short duration is much greater than with an uninterrupted exposure of equal total time. For example, more than twice as much was obtained after 8 exposures lasting one hour each, followed by an interval sufficiently long to decolourize the blue preparation, than one single exposure lasting eight hours, followed by decolouration. With intermittent irradiations for periods of 15 minutes each, the extent of reducing substance formation increased about fivefold, and with exposures lasting about 5 minutes separated by intervals sufficiently long for the blue colour to be lost, it increased about 10 times as compared with continuous radiation lasting 8 hours. On the other hand, papers treated with molybdic acid showed no appreciable increase in water soluble reducing substances even after repeated intermittent irradiation. This is in agreement with observations made with sunlight.

Mechanism of Increased Effectiveness of Intermittent Irradiation.

The increased effectiveness of repeated exposures to ultraviolet light compared with continuous irradiation, the incident radiational energy being equal in both cases, suggests the question whether the actual period of irradiation or the period of decolourization produces the greater part of the reducing substances. The difference between the experimental results obtained from tungsten in contrast to molybdenum preparations makes it reasonable to assume that the production of the water soluble reducing substances is mainly connected with the re-oxidation of the blue tungsten oxide. Further, one may assume that the water soluble reducing substances formed during irradiation as shown in the experiment with 8 hours' exposure to the rays of a mercury arc in air are due to a certain amount of reoxidation occurring during the irradiation. This is further supported by the observation that practically no water soluble reducing substances are formed during one hour of continuous irradiation. (Fig. 2.)

Without the presence of moisture the process of photolysis of cellulose does not proceed much beyond the reduction of the tungsten oxide and without interruptions of the irradiation, permitting considerable or complete reoxidation, the degradation of cellulose is considerably less.

On the basis of the experiments described one may therefore assume that the required water is decomposed and that the reduced tungsten oxide operates as an oxygen acceptor.

In comparing the photolysis of cellulose in the presence of tungstic acid with the photosynthesis of plants one notices the necessity of moisture and an oxygen acceptor in the former and of moisture and a hydrogen acceptor in the latter.

SUMMARY.

The oxides or acids of hexavalent tungsten and molybdenum when finely dispersed on dry cellulose or other polysaccharides or certain of their derivatives turn blue if exposed to light of wavelength between 230 and 390 mμ.

In the case of molybdic acid the blue colour produced by the light is retained permanently, but with tungstic acid the colouration is slowly lost in air if light of less than 390 mμ is excluded. This process of colour disappearance requires water.

The cellulose used in these experiments underwent degradation as indicated by the formation of water soluble reducing substances. For a given amount of incident radiational energy, a greater yield of water soluble reducing substances is obtained when the irradiation is intermittent than when it is applied in one continuous period. From this and other evidence it is concluded that the process of colour disappearance is mainly responsible for the production of water soluble reducing substances.

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SPECIFIC RELATION BETWEEN ACTIVE VIRUS AND THE CORRESPONDING INDICATOR STRAINS IN STUDIES OF SOLUBLE INHIBITORS

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An important advance in the study of inhibition of haemagglutination by soluble mucoids has been made by Stone's (1949a) observation that by appropriate methods any influenza virus can be converted into an "indicator" strain. By the term indicator strain we refer to a virus preparation which is inhibited by a typical mucoid such as ovomucin to a titre of the same order as is shown against the standard indicator virus, LEE heated to 56° C. for 30 minutes (Francis, 1947). The strain MELB can for instance be converted into a highly sensitive indicator by heating allantoic fluid virus diluted in citrate saline buffered at pH 8·5 for 30 minutes at 56° C. As has already been shown for heated LEE virus by Burnet (1948) and by Hirst (1948) independently, indicator viruses have no enzymic action. Hirst considered that the susceptibility to inhibition was directly due to the absence of enzymic action but we have already given reasons why this view is unacceptable at least in this form (Anderson *et al.*, 1948). In that discussion the general viewpoint adopted was that adsorption of virus to the red cell was a necessary condition for enzyme action and eventual elution, but that it was not a direct function of the enzyme and its substrate. The adsorption was ascribed to other components of the virus surface on the one hand and to the whole mucopolysaccharide aggregate making up the cell receptor on the other.

In the course of work arising out of studies on the effect of periodate on soluble inhibitors (Burnet, 1949), a number of observations were made which pointed strongly toward a more direct relationship of enzyme and substrate than that formerly envisaged. In the earlier view we regarded the enzyme as being destroyed by the manipulations used to produce an indicator strain and as taking no part whatever in the reactions of the indicator virus. In this paper a number of instances will be described in which there is a close or absolute correspondence in the specificity of enzyme action of an active virus and the specificity of inhibition of the homologous indicator strain.

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Materials and Methods.

The various reagents have been described in earlier papers. They include active virus in the form of allantoic fluid of the strains MELB, WSE and SW (Swine 15) of influenza A and LEE influenza B and mumps Strain END (Enders) and Newcastle disease virus NDV (Victorian strain). Indicator strains of the influenza viruses were prepared as described by Stone (1949a) H–LEE and H–WSE by heating allantoic fluid virus to 56° C. for 30 minutes T–MELB by diluting 1:5 in a mixture of 0·2 p.c. citrate in borate buffer pH 8·5 and heating to 56° C. for 30 minutes, T–SW by treatment for 2 hours at 37° C. similarly diluted in alkaline citrate solution.

RDE was used in the form of eluate from red cells as described by Burnet and Stone (1947a).

Inhibitor solutions included semipurified ovomucin prepared by the method of Gottschalk and Lind (1949) and two crude fluids containing natural mucin (a) from a mucocele of a human gallbladder and (b) from the ileum of the guinea pig by the method described by Burnet and Stone (1947b). Stock solutions in saline were of such concentrations as would give an inhibitory titre with H-LEE of 1,000–2,000.

Inhibitory titres were determined by the standard method of this laboratory using 5 agglutinating doses of virus with 1 p.c. fowl cells; inhibitor dilutions in normal saline and virus were left 30 minutes at room temperature before addition of the red cells which were allowed to sediment at room temperature. The end point was the standard degree of partial agglutination.

EXPERIMENTAL.

Determination of Inhibitor Gradient.

The inhibitor gradient has been defined by Stone (1949 b) from the study of the progress of enzymic action as measured by the loss of inhibitory power for different indicator viruses. It was found that for the present purposes a simpler technique was highly suitable and gave results more directly comparable with the receptor gradient of red cells. This technique has already been described in connection with work on periodate action. A suitable dilution of mucoid to give a titre of about 1–2,000 against H–LEE was prepared. To a series of 1 ml. lots of the mucoid solution 0·25 ml. of virus diluted in Ca acetate saline, pH 6·2, to 100 agglutinating units was added giving a final concentration of 20 A.U. When RDE was used 20 units as defined by Burnet and Stone (1947a) were also present in the final mixture. A drop of toluol was added as a bacteriostatic, the tubes were corked and placed overnight in a 37° C. incubator. After 17–18 hours' incubation the tubes were heated to 65° C. for 30 minutes to destroy the virus haemagglutinin and the inhibitory titres of the contents tested against the viruses required. Control mucoid without virus was always included and the titre of the indicator viruses checked in each experiment.

The results with gallbladder mucin treated with 6 viruses, Mumps NDV, MELB, WSE, LEE and SW and tested with four indicators are shown in Table 1.

The general linear character of the results is clearly visible but close examination of the Table will show that in each instance the reduction of inhibitory power is disproportionately great against the homologous indicator virus. In this instance the order of activity of the unmodified viruses is the same as the order of increasing resistance to destruction of the inhibitory action against the homologous indicator strains. With other mucoids the superadded specific effect against the homologous inhibitor may largely obscure the order of activity

of the viruses. If we confine attention to the three indicator strains which have been used most frequently, H–WSE, T–MELB and H–LEE, the order for each of the various mucoids tested in this fashion is

Ovomucin. H–WSE, H–LEE, T–SW, T–MELB.
Human gallbladder mucin. H–WSE, T–SW, T–MELB. H–LEE.
Guinea pig mucin. T–MELB = H–WSE, H–LEE.
Human blood group substance H. H–WSE, T–MELB, H–LEE.
Ovarian cyst mucoid. H–WSE, T–MELB, H–LEE.

Ovomucin differs sharply from all the mucoids of mammalian origin in the fact that the inhibitor for T–MELB is not destroyed by LEE virus. A detailed study of ovomucin in this respect will be found in Stone's (1949 b) paper.

TABLE 1.

Inhibitor gradient with a human mucin solution.

Indicator viruses	Mucins treated with active viruses						Treated RDE	Control
	Mumps	WSE	SW	MEL	NDV	LEE		
H–WSE	40	6	<2	10	7	<2	<2	600
T–SW	70	25	<2	7	5	<2	<2	300
T–MEL	1,500	1,200	100	<2	<2	2	<2	2,400
H–LEE	1,500	1,200	250	150	12	<2	<2	1,600

Figures represent the inhibitory titre as tested with each indicator virus.

Action of RDE on Guinea pig Intestinal Mucin.

In all previous work RDE has been found capable of destroying all the inhibitory power of any mucoid solution for any of the viruses. When tests for inhibitor destruction were being made with guinea pig intestinal mucin it was found that the prolonged action of RDE left a significant residue of inhibitory activity against T–MELB. This was regularly reproducible with mucin from four separate animals and the residual titre was not affected with much larger amounts of RDE. Table 2 shows the action of RDE in comparison with that of the viruses MELB and LEE.

TABLE 2.

Action of LEE, MELB and RDE on guinea pig intestinal mucin.

Mucin treated overnight with	Indicator virus		
	H–WSE	T–MEL	H–LEE
LEE	<2	32	32
MELB	5	<2	350
RDE	<2	35	<2
Control	1,600	1,500	1,000

In these experiments a titre of 30–40, i.e. 2–3 p.c. of the original inhibitory titre against T–MELB is retained after the completion of RDE and LEE action despite the fact that by ordinary criteria LEE is a more active virus than MELB on this mucin. As is usual, inhibitor against T–MEL is completely destroyed by the homologous virus. Further, if MELB virus is added to the RDE-treated material the residual activity is rapidly destroyed.

DISCUSSION.

In a detailed comparison of the action of RDE with virus action on the human red cell Stone (1947) showed that certain differences in the order of viruses in the receptor gradient appeared when different techniques were used. The most striking was the position of mumps virus which was first in the series as defined by virus action but came after NDV, MELB and WSE when progressive action of RDE was used as the criterion. The position of the three viruses at the end of the gradient BEL, SW and MIL-B also varied according to which of them was allowed to act on the cells. Differences were only slight but cells treated by one virus and subsequently stabilized were less readily agglutinated by that virus than by the others.

In our opinion the inhibitor gradients shown by Stone (1949b) for ovomucin and ovarian cyst mucoid and by a different method in the present paper for two crude mucins are entirely analogous to the cell receptor gradients and must depend on the same basic characteristics of virus-mucopolysaccharide union.

The point to be stressed from the various manifestations of specificity that have been described is that what determines susceptibility to enzyme action is apparently the same molecular pattern as determines union with the indicator form of the same virus. The example of guinea pig mucin treated with RDE is a particularly striking one. All inhibitory action has been lost except against T-MELB and the only virus which can destroy that residual activity is MELB. There seem to be only two reasonable alternatives: (1) the enzyme on the viruses plays no part whatever in adsorptive phenomena so that its destruction does not alter the specificity of adsorption in any way, or (2) the whole range of haemagglutination and inhibition reactions are manifestations of enzyme-substrate adsorption, the change to the indicator state being associated with no change in the qualitative character of the adsorption but being quantitatively firmer and lacking the capacity to induce or complete the destruction of the substrate. A discussion of these alternatives will be deferred until work on the chemical nature of the enzyme action on ovomucin in progress in this laboratory is further advanced.

SUMMARY.

A convenient method of establishing the "inhibitor gradient" for soluble mucoids is described.

Superimposed on the linear gradient there are specific effects in the sense that each active virus reduces inhibitory activity against the homologous indicator virus to an extent beyond that corresponding to its place in the gradient.

With guinea pig intestinal mucin full action of RDE leaves a residual activity against T-MELB. This is destroyed by MELB active virus.

The significance of these findings is briefly discussed.

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ENZYMATIC MECHANISMS IN THE RESPIRATION OF RHUBARB LEAVES[1]

by J. F. MORRISON

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(Accepted for publication 10th August, 1949.)

The metabolic behaviour of plants is an aspect of biochemistry that has received relatively little attention. Our meagre knowledge of plant reactions has been gained mainly from a study of the overall gas exchanges which occur in tissues subjected to various treatments, together with a few detailed investigations of the constituent enzymes, such as polyphenol oxidase of potatoes. There has been no thorough investigation of one particular plant, rather have various plants been shown to contain one particular enzyme. On the other hand, systems for the complete oxidation of metabolites have been formulated as a result of experimental work on animal tissues. The purpose of this work was to gain a general idea of the metabolism of the leaf cells, by utilizing the knowledge of animal and bacterial cell reactions, before investigating specific enzyme systems. A similar approach was made by Bonner and Wildman (1946) in their investigation of spinach leaf metabolism.

Methods.

Plants.

The rhubarb leaves were obtained from plants grown in the Brisbane Botanical Gardens, and were not picked until they had reached a reasonable stage of maturity.

Gas Exchange Measurements.

Respiration was measured in Warburg manometers at a temperature of 30° C. and at a pH value of 5·3, unless otherwise stated. Oxygen uptake and CO_2 production were determined in the usual way. The leaves were prepared by excising and discarding the main ribs and veins. The blade tissue was then folded and punched into discs of about 12 mm.^{2} and thoroughly mixed to ensure uniform sampling. Into each cup 400 mg. discs were weighed, and the buffer, together with substrates, was infiltrated into the leaf cells by the method of Bonner and Wildman (1946). This was found to have no detrimental effect on the rate of respiration.

Throughout the work, the figures shown in the Tables represent the gas uptake or evolution in mm.^{3}.

EXPERIMENTAL.

Metabolism of Leaf Discs and Homogenate.

When the leaf was homogenized with buffer, it was found to have a far slower rate of respiration than did leaf discs, when used in equivalent amounts (Table 1). This indicates the importance of cellular organisation in the metabolism of rhubarb leaf cells.

TABLE 1.

Comparison of the respiration with discs and homogenate.

Treatment	1st hour		2nd hour		3rd hour		R.Q.
	O_2	CO_2	O_2	CO_2	O_2	CO_2	
Leaf homogenate	19	10	19	11	19	11	0·58
Leaf discs	118	146	115	134	115	134	1·17

It is significant to note that the R.Q. value obtained with leaf discs is greater than unity, indicating that it is possibly organic acids which are being metabolized.

Effect of Light and Darkness on Respiration.

The oxygen uptake and the CO_2 output of leaves, in light and darkness, were determined by allowing samples of discs from the same leaf to respire either in a Warburg bath illuminated by light bulbs, or in one placed in a dark room. For the latter, light was used only when readings were taken.

It was found that there was little difference in the O_2 uptake, but there was a slight depression of the CO_2 output in light, presumably caused by the photosynthetic fixation of some of the respiratory CO_2 produced by the leaf. (Table 2).

TABLE 2.

The effect of light and darkness on respiration.

Treatment	1st hour		2nd hour		3rd hour		R.Q. 3rd hour
	O_2	CO_2	O_2	CO_2	O_2	CO_2	
Darkness	114	128	115	124	117	125	1·07
Light	112	103	114	105	105	99	0·94

Similar results were obtained with leaves picked early in the morning and those picked late in the afternoon.

A comparison of the rates of respiration of leaves picked at these times would be interesting. Unfortunately such a comparison was not possible, as great variation occurred in the amounts of O_2 taken up and CO_2 evolved in different leaves, although the rate did not vary more than 5 p.c. in duplicate samples of the same leaf.

Comparison of Qo_2 Values of Different Leaves.

In order to determine if this difference was due to variation in the water content of the leaf, 400 mg. lots were dried at 100° C. for 2 hours and the Qo_2 values determined. These values, which ranged from —0·78 to —1·92, showed that there is no correlation between the leaf dry weight and the rate of O_2 uptake.

The Effect of pH on Respiration.

Table 3 shows that the R.Q. of rhubarb leaves is influenced by the pH of the media, in which the discs are suspended. The CO_2, which is bound at pH 7·0 and 6·5, was released by the addition of dilute sulphuric acid from the side arm of the cup, at the end of the first hour.

TABLE 3.

Effect of pH on respiration.

pH	Gas exchange/400 mg. leaf/hr.			Total CO_2	R.Q.
	O_2 uptake	CO_2 output	Bound CO_2		
4·5	100	109	—	109	1·09
5·3	100	119	—	119	1·19
5·9	126	149	—	149	1·18
6·5	118	117	2	119	1·00
7·0	127	106	13	119	0·94

In all experiments it was noted that there was no consistent rate of CO_2 output or O_2 uptake by the tissue, when buffers of varying pH were used, but the R.Q. values of different leaves were reasonably constant. Bonner and Wildman (1946) obtained similar results with spinach leaves.

Cyanide.

The respiration of rhubarb leaves has been shown to be inhibited by cyanide (Table 4).

TABLE 4.

Effect of cyanide on leaf respiration.

Treatment	1st hour		2nd hour		3rd hour		Inhib. 3rd hour	
	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2
Control	164	173	144	152	176	172		
10^{-2} M KCN	44	19	34	22	31	8	82 p.c.	95 p.c.
10^{-3} M KCN	60	28	44	19	44	16	75 p.c.	91 p.c.

The fact that cyanide, at a concentration as high as 10^{-3} M, does not completely inhibit the O_2 uptake, indicates that the leaf possesses some cyanide-insensitive pathway for the uptake of oxygen. This is possibly through flavoproteins or other autoxidisable compounds. The

cyanide inhibition of respiration does, however, indicate that the majority of respiration involves a heavy metal enzyme. The greater sensitivity of the CO_2 liberating mechanism can be explained by the inhibition of the plant decarboxylases.

Azide.

The O_2 uptake and CO_2 production of the leaf was also inhibited by azide (Table 5).

TABLE 5.

Inhibition of respiration by azide.

Treatment	1st hour		2nd hour		3rd hour		Inhib. 3rd hour	
	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2
Control	125	144	122	142	107	130	—	—
10^{-2} M azide	35	13	29	9	13	9	88 p.c.	93 p.c.
10^{-3} M azide	27	12	22	7	17	6	84 p.c.	96 p.c.

At a concentration of 10^{-3} M azide seems to penetrate the cell more rapidly, but in the third hour both concentrations have practically the same inhibitory effect.

Carbon Monoxide.

Whether carbon monoxide can be satisfactorily used with intact photosynthetic tissues has not, as yet, been definitely determined. Marsh and Goddard (1939) found that the respiration of the young leaves of carrots was inhibited approximately 40 p.c. in the dark by carbon monoxide, whereas Bonner and Wildman (1946) claim that carbon monoxide cannot be used to determine the catalyst involved in the respiration of spinach leaves, because of complications inherent in a photosynthetically active tissue.

The results obtained with carbon monoxide inhibition of the leaf respiration, were most irregular and did not permit of any definite conclusions. This was unfortunate as the effect of carbon monoxide on the respiration in light and darkness, would have indicated whether cytochrome oxidase or polyphenol oxidase occurred in the leaf, as both these enzymes are inhibited by cyanide and azide.

Nitrophenols.

The O_2 uptake of the leaf was found to be inhibited 70 p.c. by 10^{-3} M p-nitrophenol, and 88 p.c. by 10^{-2} M, whereas o-nitrophenol caused only 20 p.c. inhibition of the O_2 uptake at the most, and at times there was no inhibition and even stimulation was noted (Table 6).

TABLE 6.

The effect of nitrophenols on respiration.

Treatment	1st hour		2nd hour		3rd hour		Inhib. 3rd hour	
	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2
Control	115	134	113	136	102	121	—	—
10^{-2} M p-nitrophenol	17	6	18	2	12	3	88 p.c.	98 p.c.
10^{-3} M p-nitrophenol	57	60	37	22	30	18	70 p.c.	85 p.c.
10^{-3} M o-nitrophenol	130	167	122	148	118	153	—	—

The nitrophenols can be considered as analogous to the o- and p-diphenols, which are normally oxidized by the plant tissues. The above results would indicate that p-diphenols are oxidised by the terminal oxidase, as their analogue has such a high inhibitory effect, whereas the enzyme has no action on o-diphenols. To test this hypothesis, catechol and hydroquinone were added to the leaf discs.

Catechol.

Baker and Nelson (1943) have shown that there is an initial increase in the O_2 uptake on the addition of hydroquinone and catechol to potato slices, which are known to contain polyphenol oxidase, whilst Bonner and Wildman (1946) have shown that the infiltration of spinach leaves with catechol, causes an initial rise in rate of O_2 uptake, together with a marked depression of the CO_2 production. With both tissues, there was a later fall in the rate of O_2 uptake, below that of non-infiltrated tissue. The infiltration of catechol into rhubarb leaves caused no initial rise in the rate of O_2 uptake, but there was an inhibition of the O_2 uptake which increased with time and which was dependent upon the concentration of the added catechol (Table 7).

TABLE 7.

Inhibition of respiration by catechol.

Treatment	O_2 uptake in mm^3			
	1st hour	2nd hour	3rd hour	Inhib. 3rd hour
Control	128	108	104	—
2 mg./ml.	118	76	68	35 p.c.
1 mg./ml.	116	101	93	10 p.c.
0·5 mg./ml.	124	110	108	—
0·25 mg./ml.	128	116	112	—

Using catechol at a concentration of 5 mg./ml., the degree of inhibition was found to decrease with a rise in the pH of the medium. This may have been due to a smaller amount of catechol diffusing into the cell, but it was noted in each case that there was an increase in brown colour of the contents of the cups, to which catechol had been added (Table 8).

TABLE 8.

The effect of pH on the inhibition of respiration by catechol.

pH	1st hour		2nd hour		3rd hour		Inhib. 3rd hour
	Control	C.C.	Control	C.C.	Control	C.C.	
5·3	115	65	125	57	117	47	60 p.c.
5·9	138	78	144	70	140	60	58 p.c.
6·5	126	82	127	72	126	58	54 p.c.
7·0	130	132	130	108	135	80	41 p.c.

C.C. = control and catechol.

It was earlier suggested that the inhibition of respiration by catechol was caused by the denaturing effect of o-quinone on polyphenol oxidase. Baker and Nelson (1943) have shown that the fall in the rate of O_2 uptake cannot be explained by enzyme inactivation, for, on adding 4-tertiary butyl catechol, which hardly inactivates the enzyme, to potato slices they found that the fall in O_2 uptake was greater than with catechol.

Schade, Bergmann and Byer (1948) found that with potato slices, there was a stimulation of respiration on the addition of catechol, followed by a falling off which was proportional to the initial addition of catechol. On the initial addition of relatively large amounts of catechol there was no response to further addition, whereas with smaller amounts there was a further stimulation of the rate of O_2 uptake. From these results they concluded that catechol was acting primarily as a cell poison, capable, in suitable concentrations, of destroying the processes on which the metabolic activity of the cells depend, and only secondarily as a substrate for tyrosinase. The results with rhubarb leaves indicate that catechol acts only as a cell poison and is not concerned with oxidation-reduction reactions which occur in the cell. This then excludes polyphenol oxidase as a component of respiration, for catechol has been shown to stimulate the O_2 uptake of all tissues known to contain this enzyme.

On the addition of gallic acid to leaf homogenates with buffer pH 5·3, there was no increase in the rate of O_2 uptake.

Hydroquinone.

The fact that leaf respiration was markedly inhibited by p-nitrophenol led to the idea that p-diphenols were oxidised by leaf cells. On investigation it was found that hydroquinone was similar in action to catechol, at higher concentrations causing a depression of the oxygen uptake and CO_2 output, without any stimulation (Table 9).

TABLE 9.

Effect of hydroquinone on respiration.

Treatment	1st hour		2nd hour		3rd hour		Inhib. 3rd hour	
	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2
							p.c.	p.c.
Control	110	132	112	135	107	119	—	—
5 mg./ml.	70	75	62	57	46	40	38	50
1 mg./ml.	104	124	103	121	95	109	11	9
0·5 mg./ml.	125	149	112	135	96	100	10	16

It would therefore appear that hydroquinone also acts primarily as a cell poison, when whole leaf tissue is used.

Diethyldithiocarbamate.

The marked inhibition of the respiration of the leaves by p-nitrophenol suggests that polyphenol oxidase is the terminal oxidase, although the results obtained with catechol do not lend any support. In order to determine whether a copper enzyme was involved in the respiration, diethyldithiocarbamate was infiltrated into the leaf cells and the O_2 uptake measured. (Table 10)

TABLE 10.

O_2 uptake in the presence of diethyldithiocarbamate.

Treatment	1st hour	2nd hour	Inhib. 2nd hour
Control	126	118	—
10^{-2} M D.D.C.	83	81	32 p.c.
5×10^{-3} M D.D.C.	114	112	5 p.c.
10^{-3} M D.D.C.	114	114	3 p.c.

D.D.C. = diethyldithiocarbamate.

The fact that a concentration of diethyldithiocarbamate as high as 10^{-2} M caused only approximately 30 p.c. inhibition, indicates that a copper enzyme plays no important rôle in the oxidative processes of the leaf.

Cytochrome c and p-phenylenediamine.

On the addition of cytochrome *c*, as prepared by the method of Potter (1945), there was no increase in the rate of O_2 uptake, although the preparation was found to increase the rate of O_2 uptake with the succinoxidase system of heart. p-Phenylenediamine markedly stimulated the rate of respiration but, together with cytochrome *c*, there was only a small additional increase and then only at pH 7·2 (Table 11).

TABLE 11.

The effect of cytochrome c and p-phenylenediamine on respiration.

Treatment	1st hour		2nd hour	
pH	5·3	7·2	5·3	7·2
Control	128	128	130	138
+ P.P.D.	182	190	201	227
+ cyt. c.	102	148	116	138
+ P.P.D. + cyt. c.	188	220	202	242

P.P.D. = p-phenylenediamine. cyt. c. = cytochrome *c*.

Polyphenol oxidase is not able to oxidize p-phenylenediamine as shown by Keilin and Mann (1938), whereas cytochrome oxidase is able to do so. Batelli and Stern (1914) used p-phenylenediamine as the substrate for measuring cytochrome oxidase activity. It therefore appears as though cytochrome oxidase is the enzyme involved in the terminal oxidation.

The failure of cytochrome *c* to increase the rate of O_2 uptake does not, of course, exclude participation of the cytochrome system since cytochrome *c* may already be present in optimum amounts.

p-Phenylenediamine and p-Nitrophenol.

Upon reaching the conclusion that cytochrome oxidase was present in the leaf, the marked inhibition of respiration by p-nitrophenol was left unexplained. However, p-nitrophenol can also be considered as an analogue of p-phenylenediamine, and thus the inhibitory action of the former could be explained as preventing cytochrome *c* from oxidizing p-phenylenediamine, which could possibly be the oxidation-reduction carrier in rhubarb leaf respiration.

In order to test this hypothesis p-phenylenediamine (2·5 mg./ml.) was added to the leaf in the presence of 10^{-2} M p-nitrophenol (0·6 mg./ml.), whence it was found that this compound was able to increase the inhibited rate of O_2 uptake by 100 p.c. (Table 12).

TABLE 12.

Effect of p-phenylenediamine on p-nitrophenol inhibition.

Treatment	O_2 uptake in mm^3		Inhibition 3rd hour
	1st hour	2nd hour	
Control	138	142	—
10^{-2} M p-nitrophenol	22	22	85 p.c.
10^{-2} M p-nitrophenol + P.P.D.	50	45	66 p.c.

These results therefore confirm the above hypothesis.

DISCUSSION.

Table 13 summarizes the results obtained in the determination of the terminal oxidase of the leaf.

TABLE 13.

A comparison of results obtained with inhibitors and substrates with their known action.

Inhibitor	Results obtained	Action known to occur		
		Polyphenol oxidase	Cytochrome oxidase	Ascorbic acid oxidase
KCN	+	+	+	+
Azide	+	+	+	—
p-nitrophenol	+	+	?	?
D.D.C.*	—	+	—	+
Substrates				
Catechol	—	+	+	?
P.P.D.†	+	—	+	—
Cytochrome *c*	—	—	+	—

* Diethyldithiocarbamate. † p-phenylenediamine.

The above results point to the fact that cytochrome oxidase is the enzyme concerned in the terminal oxidation. The failure of diethyldithiocarbamate to inhibit the respiration to any great extent clearly rules out the importance of the copper enzymes, polyphenol oxidase and ascorbic acid oxidase, as major respiration components. Cyanide inhibits all three oxidases. According to Bonner and Wildman (1946), p-nitrophenol does inhibit polyphenol oxidase, but whether it has any inhibitory action on cytochrome oxidase is unknown. However, the inhibition of respiration by p-dinitrophenol can be explained by considering this substance as an inhibitory analogue of the p-di-amines, which are oxidized by cytochrome oxidase through cytochrome *c*.

Support for the significance of cytochrome oxidase is given by the results obtained with the addition of substrates. Ordinarily, the cytochrome oxidase system is able to oxidize both hydroquinone and catechol, but the failure of these compounds to increase the rate of O_2 uptake with intact rhubarb leaves, can be explained by their primary rôle as inhibitors of other metabolic reactions of the cells. However, catechol does temporarily stimulate the respiration of tissues which contain polyphenol oxidase. The fact that there is no stimulation of respiration with rhubarb leaves is indicative of the absence of this enzyme and this idea is supported by the stimulatory effect of p-phenylenediamine, which is not oxidized by polyphenol oxidase. On the other hand, p-phenylenediamine is oxidized by cytochrome oxidase, and this oxidation could be inhibited by the structurally related p-nitrophenol. The failure of cytochrome *c* to increase the rate of respiration may have been due to the amount of cytochrome oxidase being the limiting factor.

Although it is concluded that one of the known oxidases occurs in the leaf, it is possible that the conclusions reached as a result of the effects of inhibitors and substrates on respiration, are not valid. As plants possess a number of phenolic compounds and pigments, it is possible that these compounds play a rôle in oxidative reactions, that some of the sites of action of inhibitors are as yet unknown and that catechol and hydroquinone could function by breaking a series of chain reactions, thus causing a decrease in the rate of O_2 uptake.

SUMMARY.

The respiration of rhubarb leaf discs has been studied by gas exchange measurements after various treatments.

The rate of respiration varied with different leaves and was decreased by homogenization.

The R.Q. value of leaves decreased in the presence of light, owing to a fall in the CO_2 production. The same effect was obtained when the pH of the medium was increased.

Cyanide, azide and p-nitrophenol inhibited the rate of O_2 uptake, but diethyldithiocarbamate and o-nitrophenol did not cause any appreciable effect.

Both catechol and hydroquinone inhibited the rate of O_2 uptake, without any initial stimulation. Cytochrome *c* was not able further to increase the increased rate of O_2 uptake with p-phenylenediamine.

From the above results it was concluded that the leaf contains cytochrome oxidase or some closely related terminal oxidase.

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THE THERMAL DESTRUCTION OF VITAMIN B_1

FURTHER STUDIES ON THE INFLUENCE OF THE CONCENTRATION OF BUFFER SALTS ON THE RATES OF DESTRUCTION OF ANEURIN AND COCARBOXYLASE

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(Accepted for publication 18th July, 1949.)

In an earlier paper (Farrer, 1947 b) the present author has discussed the effect of the concentration of the buffer salts on the rate of destruction of aneurin. This paper relates primarily to cocarboxylase, the rate of destruction of which is affected in a similar way to that of aneurin under the same conditions of buffer concentration. But arising out of this work it has been found that the earlier statement that the concentration of McIlvaine citric acid-phosphate solutions is without effect on the rate of destruction of aneurin is wrong. This concentration effect is described and a probable explanation for the earlier erroneous conclusion is given. It has also been necessary to modify slightly the pH/log k curve for aneurin destruction in M/5 phosphate-phthalate solutions between pH 5·5 and 6·2 and in M/50 solutions between pH 4·7 and 5·0.

EXPERIMENTAL.

The reagents, assay method and experimental procedure are similar to those used in earlier papers of this series.

Reagents:

Buffer Salts. The following solutions were prepared from A.R. reagents and glass distilled water and freed from heavy metals by the $CaCO_3$ adsorption method of Stout and Arnon (1939): (i) a solution M/2 with respect to both potassium hydrogen phthalate and K_2HPO_4; (ii) M/2 Na_2HPO_4. Several batches of each solution were used during this work. The purified solutions were filtered and, in all cases except that of the Na_2HPO_4 solutions used for the McIlvaine buffer solutions (q.v.) a measured volume of the filtrate was just acidified with the 6 N HCl (q.v.) and boiled to expel CO_2. When cool the solution was adjusted to the original volume with twice distilled water.

Citric Acid. As previously recorded (Farrer, 1947 a) pure solutions of citric acid cannot be obtained satisfactorily by $CaCO_3$ adsorption. A.R. citric acid was therefore successively recrystallized from twice distilled water until the solid crystals showed considerably less than 1 p.p.m. of heavy metals in the dithizone test. M/10 solutions were prepared and the concentration checked by titration.

6 N Hydrochloric Acid. HCl (A.R.) was twice distilled from all glass apparatus.

Twice distilled water. Doubly distilled water was prepared in all glass apparatus with a "strip action" still. It was used in the preparation of all solutions.

Aneurin stock solution. Pure aneurin hydrochloride (10 mg.) was dissolved in twice distilled water, acidified with a drop or two of the 6 N HCl and the solution diluted to 500 ml. 1 ml. $\equiv$ 20μg. of aneurin.

Cocarboxylase stock solution. 10 mg. of synthetic cocarboxylase (Merck & Co., Rahway, N.J.) were dissolved in twice distilled water, slightly acidified with 6 N HCl and the volume made up to 250 ml. 1 ml. $\equiv$ 40 μg. of cocarboxylase.

Method.

The Jansen thiochrome method was used as described earlier (Farrer, 1945 a); direct assay with aneurin and assay after overnight incubation with taka-diastase at pH 4·5 and 37° C. to hydrolyse cocarboxylase to free aneurin. The fluorescence was read by means of a Coleman Photo-electric Fluorimeter, Model 12A.

Procedure.

In the case of the phosphate-phthalate and phosphate buffers, the appropriate amount of purified solution was pipetted into a 200 ml. volumetric flask. 10 ml. of aneurin or 5 ml. of cocarboxylase stock solutions were added (final concentration = 1 μg./ml.) and, after dilution with twice distilled water, the pH was adjusted to the desired value with 6 N HCl using the glass electrode. The solution was then made up to volume and transferred to the boiling flask which was fitted to a reflux condenser by means of a ground glass joint. As the solution boiled, samples were withdrawn at regular time intervals and assayed by the thiochrome method (the cocarboxylase after hydrolysis as already mentioned). At the conclusion of each experiment the pH was re-checked.

The McIlvaine citric acid-phosphate buffers are prepared by adding x ml. of M/10 citric acid to y ml. of M/5 Na_2HPO_4 (cf. Britten, 1932). The pH is varied by varying x and y. It is thus impossible to use acidified Na_2HPO_4 without interfering with the citric-phosphate ratio for the buffers of pH values below 6·5. Purified, unacidified solutions of Na_2HPO_4 (M/2) were, therefore, used. The exact amount of citric acid corresponding with the desired pH was pipetted out and the cocarboxylase (or aneurin) and rather less than the calculated amount of phosphate solution were added. The solution was shaken very thoroughly, sometimes with warming, to expel CO_2 (from traces of dissolved $CaCO_3$ in the unacidified, purified Na_2HPO_4) and diluted with twice distilled water. The pH was adjusted to the desired value with the phosphate solution and the volume made up to 200 ml. with twice distilled water. The use of the exact amount of citric acid (x ml.) and the addition of a more concentrated solution of Na_2HPO_4 to give the correct pH ensured the presence of the equivalent of y ml. of M/5 Na_2HPO_4. The small residual amounts of CO_2 in the final additions of Na_2HPO_4 were driven off in the subsequent boiling but were without effect on the pH.

Results.

As in the earlier work, the galvanometer readings are equivalent to $(a-x)$ in the monomolecular reaction equation and $\log_{10}(a-x)$ was plotted against time. From the resulting straight line, k, the velocity coefficient, has been calculated for each experiment. Figs. 1, 2, 4, 5 and 7 relate log k and pH, Figs. 3 and 6 k and concentration of buffer salts for the various experimental conditions described.

The first studies were those of the destruction of cocarboxylase in M/5 and M/50 phosphate-phthalate solutions. The log k/pH curves are shown in Fig. 1. It is quite apparent that cocarboxylase destruction is slower in the more dilute buffer solution as is that of aneurin (Farrer, 1947 b). Moreover, Δ log k, i.e. the difference between log k for cocarboxylase and log k for aneurin is approximately the same at each dilution, increasing with pH (cf. Farrer, 1945 b). The first comparisons of Δ log k with the "theoretical" figure based on the earlier work (which suggests that Δ log k varies with pH only) showed some anomalies in the aneurin curves obtained (Farrer, 1947 b): at about pH 5·5 in M/5 solutions and 4·8 in M/50 solutions. Careful repetition of experiments with aneurin gave the corrections shown in Fig. 2 and the "correct" Δ log k values.

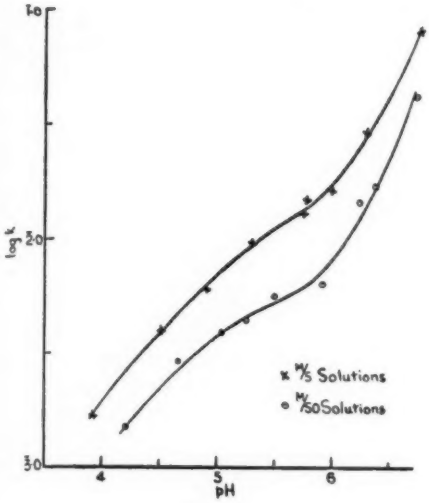

Fig. 1.

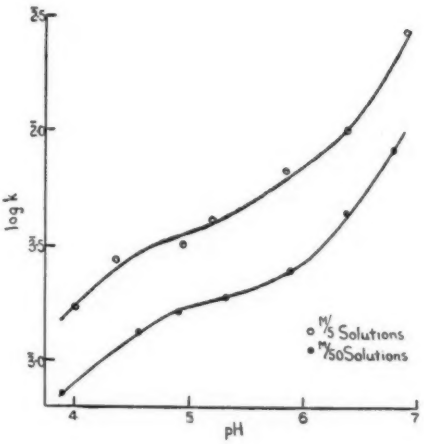

Fig. 2.

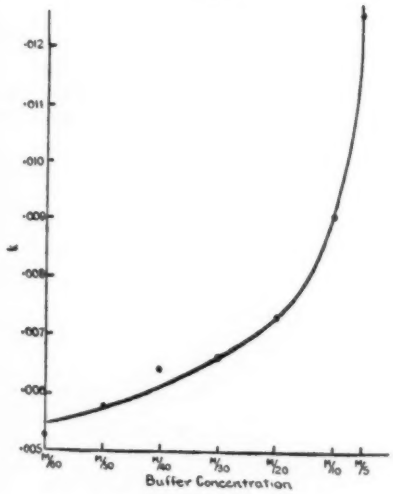

Fig. 3.

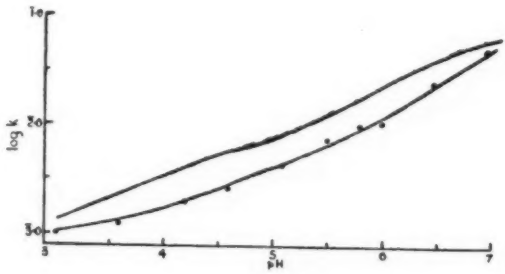

Fig. 4.

Fig. 1. log k/pH curves for the destruction of cocarboxylase at 100° in potassium phosphate-potassium phthalate buffer solutions.

Fig. 2. log k/pH curves for the destruction of aneurin at 100° in phosphate-phthalate buffer solutions: amended from Farrer (1947 b).

Fig. 3. k/concentration curve for destruction of cocarboxylase at 100° in phosphate-phthalate solutions at pH 5·75 ± 0·03.

Fig. 4. log k/pH curve for cocarboxylase destruction at 100° in citric acid-phosphate buffer of $\frac{1}{10}$th the normal concentration compared with the curve obtained by Farrer (1945) at the normal McIlvaine buffer concentration.

By varying the concentration of the phosphate-phthalate solutions the curve shown in Fig. 3 was obtained. This is substantially the same as that obtained for aneurin (Farrer, 1947 b) but at a higher level, although the cocarboxylase studies were done at a slightly lower pH, 5·75 as against 5·90 for aneurin. k is little affected by the concentration up to M/30 but thereafter, as the concentration increases, k rapidly increases.

In the earlier paper it was reported that the log k/pH curve for aneurin in McIlvaine citric acid-phosphate buffers was the same at the normal concentration (i.e. in solutions prepared by mixing varying quantities of M/10 citric acid and M/5 Na_2HPO_4) and at one tenth this concentration. This statement was based on a series of experiments at eight different pH values between 3·0 and 7·1 and it was not therefore anticipated that there would be any difference between the cocarboxylase curves obtained at the same two concentrations of buffer. However, the rate of cocarboxylase destruction was found to be less in the more dilute solution (see Fig. 4) whereupon the log k/pH curve for aneurin in the diluted McIlvaine buffer solutions was carefully repeated. This time it was found that aneurin destruction was slower in the more dilute solutions (Fig. 5) and k was determined at various concentrations of buffer solution at pH 6·0. The curve obtained is shown in Fig. 6 and it is seen that small increases in concentration at first lead to large increases in reaction velocity which rapidly becomes almost constant. In this would seem to be the explanation for the earlier erroneous statement as apparently what were thought to be solutions of one tenth the normal concentration were in fact sufficiently more concentrated than this (probably through volume loss on autoclaving) to raise k to a value which appeared to be within experimental error of that corresponding with the log k/pH curve already obtained for aneurin in the undiluted buffer. The k/concentration curve for cocarboxylase is similar to that for aneurin, but at a higher level of k.

Fig. 7 shows the relation of the rate of cocarboxylase destruction in M/50 solutions of sodium phosphate to the log k/pH curve obtained earlier (Farrer, 1945 b) for M/15 solutions of phosphate. The effect of the lower concentration on cocarboxylase destruction is closely parallel to that on aneurin destruction (Farrer, 1947 b) disappearing, as in the latter case, as the pH rises.

DISCUSSION.

From this work it is apparent that cocarboxylase is subject to the same concentration effect as aneurin in that in lower concentrations of buffer salts the hydrolysis of the molecule is lessened. Phosphate ions show the smallest concentration effect. Citrate ions, contrary to the earlier statement, do have a small effect in that the rate of destruction of cocarboxylase is less, over a range of pH values, in the more dilute solutions of the citric acid-phosphate buffer. The most significant effect is that of the phthalate ions, the solutions M/50 with respect to phosphate and phthalate giving rise to much slower rates of destruction of cocarboxylase. In all three cases the log k/pH curves are similar to those obtained for aneurin, the more dilute solutions giving slower rates of destruction. Similarly, in the two buffers studied, increasing concentrations of buffer salts produce the same effect on the reaction velocity for cocarboxylase as for aneurin. The only difference between the behaviour of the two forms of vitamin B_1 is that noted earlier, viz., that in any given set of conditions cocarboxylase is destroyed more rapidly than aneurin by an amount which is roughly proportional to the pH.

From the curves obtained for the dilute buffer solutions Δ log k has been

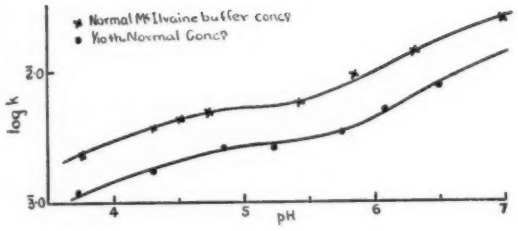

Fig. 5.

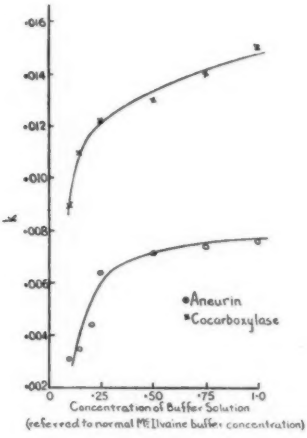

Fig. 6.

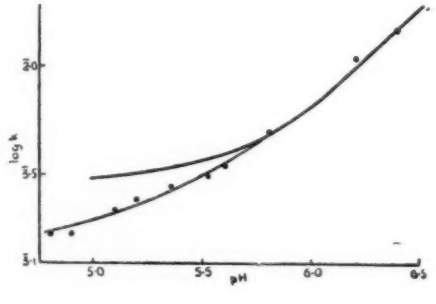

Fig. 7.

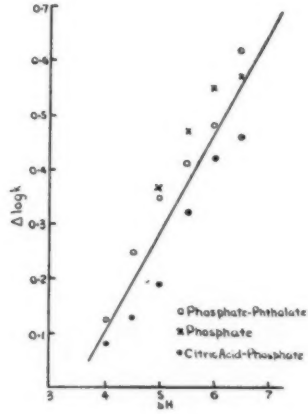

Fig. 8.

Fig. 5. log k/pH curves for aneurin destruction at 100° C. in citric acid-phosphate buffer curves of different concentrations.

Fig. 6. k/concentration curve for destruction of aneurin and cocarboxylase at 100° C. in citric acid-phosphate buffer solutions at pH 6·0 ± ·03.

Fig. 7. log k/pH curve for destruction of cocarboxylase at 100° C. in M/50 phosphate solution compared with the curve obtained by Farrer (1945) for M/15 solutions.

Fig. 8. Values of Δ log k obtained with dilute (M/50) buffer solutions compared with the Δ log k/pH curve obtained by Farrer (1945).

computed over a range of pH values and compared with the linear curve obtained earlier (Farrer, 1945 b) (see Fig. 8). The general linear relation between Δ log k and pH still holds but apparently ionic variations begin to appear in this relationship also at the lower concentrations. Thus values of Δ log k for M/50 phosphate and M/50 phosphate-phthalate solutions are much higher than any obtained for the more concentrated solutions and those for the citric acid-phosphate solutions at one-tenth normal concentration are much lower (cf. the divergence between the two curves in Fig. 6). There was in the more concentrated solutions an indication of an ionic effect in the Δ log k/pH relationship in a slightly sigmoid curve for the values of Δ log k obtained from the citric acid-phosphate solution. This same shape is evident in the values found in the more dilute solutions of the same buffer.

This work, then, emphasizes the greater vulnerability of cocarboxylase. Because of the parallel behaviour of aneurin and cocarboxylase it suggests that the same influences operate, and, by inference, that the faster destruction of cocarboxylase is linked with the phosphate grouping which constitutes the only difference between the two molecules. In addition this work underlines the importance of the varying catalytic effects of the varying anions.

SUMMARY.

Further kinetic studies of the thermal destruction of aneurin and cocarboxylase have been carried out in phosphate, phosphate-phthalate and citric acid-phosphate solutions of varying concentration and pH.

In all three buffer solutions greater dilution has brought about slower rates of destruction of cocarboxylase analogous to a similar concentration effect with aneurin.

The relationship between the rate of destruction of cocarboxylase and concentration of buffer salts varies with the buffer used but again parallels the same relationship for aneurin.

The difference between the reaction velocities for the thermal destruction of cocarboxylase and aneurin was again found to be roughly proportional to the pH but is not entirely independent of the ionic environment at the lowest concentrations (M/50) of buffer salts studied.

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THE OCCURRENCE OF SALMONELLA TYPES IN AUSTRALIA[1]

5. *SALMONELLA BLEGDAM*

by NANCY ATKINSON, GWENDOLYN M. WOODROOFE, A. M. MACBETH, HELEN CHIBNALL AND SUZANNE MANDER

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(Accepted for publication 3rd September, 1949.)

In 1944 Atkinson, Woodroofe and Macbeth recorded the first four isolations of *S. blegdam* from cases of enteric-like fever occurring in Australian soldiers in New Guinea. Later Fenner and Jackson (1946) described about 50 cases, not all confirmed bacteriologically, of *S. blegdam* infection in our soldiers in New Guinea, and Atkinson (1946) provided a preliminary report on 25 strains of the organism of which some came from these cases, and others from infections in natives. Subsequently Stevens (1946) described the occurrence of *S. blegdam* infection in the Philippines and Jones and Fenner (1947) gave an account of 14 cases in natives of New Guinea and the Solomon Islands and two more cases in Australian services personnel.

The present report gives the results of detailed investigation of 42 local strains of *S. blegdam*, the majority of which have been recorded by Atkinson *et al.* (1944, 1947, 1949). Fenner and Jackson (1946) and Jones and Fenner (1947) provided histories for most of the cases from which these strains came. This first large group of strains supplies valuable results for the characterization of this organism of which one strain only was available for Kauffmann (1935) to describe; and only one subsequent isolation (which was wrongly named *S. panama* according to Kauffmann, 1941) appears to have been recorded until our isolations in 1944. Our work establishes *S. blegdam* as an undoubted human pathogen usually producing severe enteric-like fever frequently associated with complications such as cystitis or other suppurative processes, not commonly found in enteric fever itself. We also included in our study the type culture of *S. blegdam*, two strains from New Orleans, U.S.A., kindly supplied by Dr. Erich Seligmann, and a strain, for which we are indebted to Dr. J. H. Bragdon through Dr. Frank Fenner, from an American soldier infected in New Guinea. This strain was previously identified elsewhere as

[1] This work was partly supported by a grant from the National Health and Medical Research Council.

S. enteritidis (Baker and Bragdon, 1947). The two cases from which Dr. Seligmann's strains came were described by Holt and Newton (1948).

Australian troops infected with *S. blegdam* had received routine T.A.B. vaccination which had failed to protect them against this organism in spite of the superficial similarity of its somatic antigens to those of *S. typhi*. This failure suggested that *S. blegdam* might possess factors associated with virulence which were different from those of *S. typhi*. Typhoid Vi phages failed to lyse our strains of *S. blegdam*, the virulence of which appeared to be related to susceptibility to a paratyphoid B Vi phage which we adapted to *S. blegdam*.

Antigenic Analysis.

All cultures (with the exception of N.XIV) appeared smooth by colonial appearance and by testing with trypaflavine. The following results refer to the smooth cultures. In slide agglutination tests they reacted strongly with O antiserum for Group D of the Kauffmann-White classification. The organisms were monophasic and their H suspensions agglutinated with H antisera produced against all Salmonellas containing the H antigens g or m. To confirm the presence of factor m and to detect the presence of any of the other factors f, p, u and q which may be associated with g in Group D, absorbed H antisera were prepared as follows:

For factor f—*S. derby* antiserum absorbed twice with *S. essen* and *S. budapest*.
For factor m—*S. oranienburg* antiserum absorbed twice with *S. senftenberg* and *S. berta*.
For factor p—*S. dublin* antiserum absorbed twice with *S. enteritidis*.
For factor q—*S. moscow* antiserum absorbed twice with *S. enteritidis*.
For factor u—*S. rostock* antiserum absorbed twice with *S. dublin*.

H suspensions of our cultures agglutinated with the absorbed antisera for factors m and q but failed to agglutinate with the others. Thus these cultures appeared to be *S. blegdam* and mirror agglutinin absorption tests with *S. blegdam* and its homologous antiserum and representative members of our group and their homologous antisera confirmed this identification. *S. blegdam* antiserum after absorption with a strain from our group failed to agglutinate either H or O suspensions of *S. blegdam*. The antiserum prepared against our cultures and absorbed with *S. blegdam* no longer agglutinated H or O suspensions of our organisms. Strain Bragdon which had been typed elsewhere as *S. enteritidis* reacted as *S. blegdam* in these tests and was submitted to an extra confirmatory test also. It was used to absorb *S. moscow* antiserum from which it removed all H agglutinins for *S. moscow*. This result further confirmed the presence of factor q and the identity of the strain as *S. blegdam*.

Strain N.XIV was rough when received and therefore presented difficulty in serological typing. Suspensions were unstable and direct agglutination tests were unsatisfactory. However, the highly characteristic H antigens gmq appeared to be present as absorption of *S. blegdam* antiserum with N.XIV removed all H agglutinins for *S. blegdam*. To obtain further evidence an H antiserum was prepared against N.XIV and absorbed with *S. enteritidis*. This absorbed serum still agglutinated *S. blegdam*. We therefore considered that N.XIV was a rough variant of *S. blegdam*.

Biochemical Reactions.

All strains were submitted to the various tests used by Kauffmann (1935) in his investigation of the enteritidis group; the method of testing followed either that of Kauffman (1941) or that of Edwards and Bruner (1940). Some results are given in Table 1.

TABLE 1.

Biochemical reactions of strains of S. blegdam.

Strain Number or Name	Number Tested	Peptone Water			Stern	H_2S	Gel.	Bacto-peptone sol.			Bitter solution				Simmons agar			
		dul.	inos.	xyl.	Glycerol		liq.	cit.	mue.	d.-tart.	gluc.	arab.	dul.	rham.	gluc.	arab.	dul.	rham.
†VII–N.XIII and Jap. P.O.W.–G.P.H.B.	44	+3–4	—	+1	+	+1	—	+2–4	+1	+2–4	+	+	—	+	+1–4	+2–6	—	+2–6 (2 strains —)
N.XIV	1	+3	—	+1	+	+1	—	+2	+1	+2	—	—	—	—	+2	—	—	+6
S. blegdam (type culture)	1	+3	—	+1	+	+1	—	+2	+1	+2	+	+	—	+	+1	+3	—	+3
S. blegdam 1*	1	+3	—	+1	+	+	—	+1–2	+1	+1	+	+	—	+	+1	+1	—+3	+2
S. blegdam 2*	1	+3	—	+1	+	+	—	+1–2	+1	+1	—	—	—	—	—	—	—	—

Under heading "peptone water" + indicates acid and gas production; elsewhere it represents a positive reaction to the test as indicated by the authors. The figures accompanying this sign indicate the number of days required for the positive reaction to appear.

* The results for these two strains are taken from Kauffmann (1941) and included for comparison.

dul. = dulcitol
inos. = inositol
xyl. = xylose
gel. liq. = gelatine liquefaction
cit. = citrate
mue. = mucic acid
d-tart. = d-tartrate
gluc. = glucose
arab = arabinose
rham. = rhamnose

† The strain numbers are those given in full in Table 2.

In peptone water all our strains and the type culture produced acid and gas within 24 hours at 37° C. from glucose, mannitol, xylose, sorbitol and maltose. Dulcitol was fermented more slowly than the other fermentable substrates, but all strains, including the type culture, produced acid and gas within four days at 37° C. and more than half the strains had already commenced to ferment the dulcitol after three days. Changes in pH of the medium were followed for representative strains. With glucose, mannitol, xylose, sorbitol and maltose, acid quickly appeared and the culture fluid reached pH 5–4·5 in 24 hours. This acidity was maintained and many of the cultures had died after 14 days. With dulcitol acidity appeared more slowly and reached about pH 5 after four days. After 14 days the pH of some cultures had returned to approximately 7. In 38 days at 37° C. no strain had fermented adonitol, salicin, sucrose, lactose or inositol. A purple-lilac colour appeared in the Stern glycerol test with all strains within 8 days at 37° C. This result corresponded to the reaction recorded as + by Kauffmann (1941). The Bitter test was positive for all strains except N.XIV with glucose, arabinose and rhamnose; with dulcitol all strains were negative. Strain N.XIV gave a negative reaction in all Bitter tests. On Simmons agar with glucose all strains grew well and produced a bright yellow colour within four days. On this medium growth was slower with arabinose or rhamnose but all strains except three had grown within six days. The three exceptions were strains XLV, XIII and N.XIV. No growth occurred with dulcitol within six days. In Bacto-peptone solution all strains attacked mucic acid in 24 hours, and d-tartrate and citrate within four days.

None of the strains formed indol in peptone water during five days at 37° C. None liquefied gelatine during 30 days at 16° C. On beef extract agar all strains produced abundant H_2S, but none produced ammonia (detectable by Nessler's reagent) in peptone water incubated for 24 hours at 22° C. or 37° C. or for five days at 37° C. Reference to Table 1 shows that the majority of our strains closely resembled Kauffmann's *S. blegdam* 1. The only significant discrepancy occurred with dulcitol in Simmons agar in which our strains all failed to grow in six days whereas Kauffmann's *S. blegdam* 1 grew in three days. Our strain N.XIV resembled Kauffmann's *S. blegdam* 2 in its failure to react in the Bitter tests but differed in slowly producing growth on Simmons agar with glucose or rhamnose.

On sheep or human blood agar haemolysis was not produced, but on rabbit blood agar a diffuse clearing occurred where the growth was heavy. No haemolysis of sheep, human or rabbit blood occurred in broth cultures during 48 hours at 37° C. Also broth cultures, 24 hours old, were mixed with a 2 p.c. suspension of sheep, human, rabbit or chicken erythrocytes and failed to produce haemolysis in one hour at 37° C. but the colour of the cells changed to purple. Broth cultures mixed with lysed rabbit cells also became purple and spectroscopic examination showed the presence of reduced haemoglobin. On shaking, the purple colour changed to red and spectroscopically oxyhaemoglobin was then present. The reducing activity depended upon the presence of living *S. blegdam* in broth, peptone water or meat extract and appeared not to be due to reducing substances present in the culture medium, as saline suspensions of *S. blegdam* or supernatant fluids from broth cultures were inactive. Broth cultures also readily reduced methylene blue. Some non-haemolytic staphylococci produce haemolysis of erythocytes in the hazy zones surrounding β-toxin-forming staphylococcal colonies on sheep blood agar (Christie and Graydon, 1941). On this medium no haemolysis occurred around colonies of *S. blegdam* growing in the β-toxin zone of staphylococcal colonies. Thus no definite haemolytic activity was demonstrated for *S. blegdam*.

Representative strains were tested for coagulase and fibrinolysin production. The coagulase test was performed by the slide and tube methods according to Cadness-Graves *et al.* (1943) and gave negative results. To test for fibrinolysin the method devised by Tillett and Garner (1933) for haemolytic streptococci was used and no dissolution of the plasma clot occurred. According to its authors this method was not always reliable for detection of

staphylococcal fibrinolysin which was readily demonstrated, however, by Christie and Wilson (1941) on fibrinogen agar. Strains of *S. blegdam* were therefore tested by this second method and again found negative.

Habitat and Pathogenicity.

The sources of our 45 strains of *S. blegdam* are presented in Table 2.

All except seven strains came from human material and were mainly associated with severe illness. The seven exceptions (F.M., M.1–M.5 and G.P.H.B.) came from six mice and one guinea-pig examined during an epidemic in these animal stocks in an Army laboratory in Victoria, Australia. Of the 38 human strains, 35 (VII–LI, Gray, McCall, Duncan, N.I–N.XIV, Jap. P.O.W. and Bragdon) came from cases occurring in New Guinea or Bougainville Island, one (LII) came from the only case possibly infected in Australia, and two (Seligmann A.4119 and B.4168) from cases occurring in New Orleans, U.S.A. In the New Guinea group 21 cases (VII–LI, Gray, McCall and Duncan) occurred in Australian Army personnel, 12 (N.I–N.XIV) in natives, one (Jap. P.O.W.) in a Japanese prisoner-of-war and one (Bragdon) in an American soldier. The majority of the Australian Army cases and all the native cases have been described by Fenner and Jackson (1946) and Jones and Fenner (1947) whose case numbers are used to designate our strains and appear in Table 2. Not all the cases described were confirmed bacteriologically and therefore the numbers in the Table are not consecutive but refer only to those cases from which *S. blegdam* was isolated. Three confirmed cases were not described elsewhere and they are designated as Gray, McCall and Duncan. The strain Bragdon was sent to us for typing by Dr. Frank Fenner who obtained it from Dr. H. J. Bragdon. It was isolated from one of six cases (but the specific case was not stated) described by Baker and Bragdon (1947) in American soldiers in New Guinea. Of these six cases five were bacteriologically confirmed and the organism was identified at the time as *S. enteritidis*.

The chief manifestation of *S. blegdam* infection in man was an enteric-like fever, described by Fenner and Jackson (1946), in which the organism tended to localize and cause suppuration. Of their 52 cases in Australian Army personnel 22 suffered uncomplicated typhoid-like illness, 28 commenced similarly but developed a localization of the infection in various parts of the body producing complications such as cystitis, which was fairly common, or more rarely cholecystitis, bronchopneumonia and pericarditis. One patient had diarrhoea as the only manifestation and one case suffered a typical gastro-enteritis. Five of the six American Army cases of Baker and Bragdon (1947) were described as similar to typhoid fever and all developed focal suppuration. The 14 cases of Jones and Fenner (1947), in natives of New Guinea and the Solomon Islands, consisted of four typhoid-like fevers, five septicaemias with various local manifestations, two presenting with abscesses, one gastro-enteritis, one healthy carrier and one unclassifiable on the information available. Stevens (1946) gave only a brief mention of four cases from which *S. blegdam* was isolated in the Philippines but these appeared to resemble ours. Three occurred in American soldiers of whom two showed "symptoms of paratyphoid infection with the organism detected in the blood," and one "with no apparent enteric fever" provided a strain of *S. blegdam* from faeces. The fourth case was a Filipino suffering from an ulcerative lesion of the ankle. Enteric-like fever, complicated by erythema was found in one of the two recent cases of Holt and Newton (1948) in New Orleans, U.S.A. The other case in which *S. blegdam* was isolated from blood showed upper respiratory and gastro-intestinal symptoms likened by the authors to those of the patient from whom *S. blegdam* was first isolated (Kauffmann, 1935).

In the literature the total number of strains of *S. blegdam* recorded seems to be 55. The occurrence of these strains and the references to them are given in Table 3.

TABLE 2.

Sources of 45 strains of S. blegdam.

Case and strain number[1] or name	Patients or animals infected with *S. blegdam*	Specimens containing *S. blegdam*	Type of illness
VII	Australian soldier, New Guinea	Faeces	Enteric-like fever, uncomplicated
VIII	Australian soldier, New Guinea	Blood	Enteric-like fever, uncomplicated
X	Australian soldier from New Guinea who became ill shortly after returning to South Australia on leave.	Blood, faeces	Severe enteric-like fever, uncomplicated
XII	Australian soldier, New Guinea	Blood	Enteric-like fever, uncomplicated
XIII	Australian soldier, New Guinea	Blood	Enteric-like fever, uncomplicated
XIX	Australian soldier, New Guinea	Faeces	Enteric-like fever, uncomplicated
XXIII	Australian soldier, New Guinea	Blood	Enteric-like fever, uncomplicated
XXV	Australian soldier, New Guinea	Blood, faeces	Enteric-like fever with cystitis
XXIX	Australian soldier, New Guinea	Blood, faeces, urine	Enteric-like fever with cystitis and epididymo-orchitis
XXX	Australian soldier, New Guinea	Blood	Enteric-like fever with broncho-pneumonia and pleural effusion
XXXVIII	Australian soldier, New Guinea	Blood, faeces, urine	Fatal enteric-like fever with cystitis and broncho-pneumonia
XXXIX	Australian soldier, New Guinea	Faeces, bile	Enteric-like fever with recurrent cholecystitis; *S. blegdam* detected in faeces for at least 8 months; patient discharged as a carrier
XLI	Australian soldier, New Guinea	Faeces	Enteric-like fever with epididymo-orchitis
XLII	Australian soldier, New Guinea	Faeces, bile	Fever, vomiting, empyema of gall-bladder; *S. blegdam* detected in faeces for about 12 months; carrier state cleared up by cholecystectomy.
XLIII	Australian soldier, New Guinea	Urine, faeces	Enteric-like fever, cystitis and recurrence of fever.
XLV	Australian soldier, New Guinea	Blood, faeces, urine	Enteric-like fever, cystitis and recurrence of fever, epididymo-orchitis.
XLIX	Australian soldier, New Guinea	Blood, pericardial fluid	Fever and jaundice, pneumonia, pericarditis and pleural effusion
LI	Member of A.A.M.W.S., Morotai	Faeces	Slight fever, abdominal pain and vomiting lasting 4 days
LII	Australian soldier in N.S.W., Australia, who had left New Guinea six weeks previously.	Blood, urine	Severe enteric-like fever and cystitis
Gray	Australian soldier from New Guinea	Faeces, post mortem heart blood and bile	Traumatic rupture of liver, high fever and enteritis
McCall	Australian soldier from New Guinea	Pus from empyema	Gun-shot wound of the chest with empyema
Duncan	Presumably an Australian soldier in New Guinea as the culture came with a large batch from there, but no exact information could be obtained	No information available	No information obtainable
N.I	Native adult male in a native labour camp, Port Moresby	Rectal swab	Healthy individual
N.II	Native male child, Bougainville Island	Faeces	Fever and diarrhoea subsiding after a few days but recurring in 16 days
N.V	Native adult male, Dobadura	Pus	Abscess of thigh
N.VI	Native adult female, Bougainville Island	Pus	Diarrhoea, abscess of the breast, the patient died

TABLE 2 (continued).

TABLE 2 (continued).

Case and strain number[1] or name	Patients or animals infected with *S. blegdam*	Specimens containing *S. blegdam*	Type of illness
N.VII	Native adult male, Lae	Blood	Fever, pneumonia, and slight jaundice
N.VIII	Native adult male, Lae	Blood, post mortem spleen and colon	High fever, haemolytic anaemia and jaundice, the patient died
N.IX	Native adult male, Lae	Faeces, urine, post mortem spleen	Fever, haemolytic anaemia, jaundice, cystitis, the patient died
N.X	Native adult male, Lae	Blood, post mortem spleen	High fever, jaundice, patient became comatose and died
N.XI	Native adult male, Lae	Post mortem spleen and kidney	Fever and cystitis, patient became comatose and died
N.XII	Native adult female, Bougainville Island	Urine, post mortem lung, spleen, bowel, kidney	Fever and cystitis, patient died after prolonged febrile illness
N.XIII	Native child, Bougainville Island	Post mortem small bowel	Constipation unrelieved by enemata or purgatives, patient died and only abnormal post mortem feature was the total collapse and flattening of 6 inches of small bowel which was gangrenous
N.XIV	Native adult female, Bougainville Island	Blood, urine, post mortem spleen, kidney	Fever and cystitis, patient died after prolonged febrile illness
Jap. P.O.W.	Japanese prisoner of war, New Guinea	No information available	Probably enteric-like fever, patient died
Bragdon[2]	American soldier, New Guinea	No information available	One of six cases of fever or abscess formation
Seligmann A4119[3]	Civilian female, New Orleans, U.S.A.	Faeces or blood	Enteric-like fever with erythema; or gastro-enteritis and symptoms of involvement of upper respiratory tract
Seligmann B4168[3]	Civilian female, New Orleans, U.S.A.		
F.M.	Mouse from laboratory stock, Victoria, Australia	Spleen	Epidemic disease killing many mice in the stock of a single laboratory
M.1	Mouse from laboratory stock, Victoria, Australia		
M.2	Mouse from laboratory stock, Victoria, Australia		
M.3	Mouse from laboratory stock, Victoria, Australia		
M.4	Mouse from laboratory stock, Victoria, Australia		
M.5	Mouse from laboratory stock, Victoria, Australia		
G.P.H.B.	Guinea-pig from laboratory stock, Victoria, Australia	Heart blood	Epidemic disease in stock in same laboratory at about the same time as the mouse epidemic

[1] The numbers are those of the cases recorded by Fenner and Jackson (1946) and Jones and Fenner (1948).

[2] This strain was sent to us by Dr. Frank Fenner who received it from Dr. J. H. Bragdon; it came from one of the six cases described as infections with *S. enteritidis* by Baker and Bragdon (1947).

[3] These strains were kindly sent by Dr. Erich Seligmann and were isolated from two cases described by Holt and Newton (1948).

TABLE 3.

Occurrence of strains of S. blegdam.

Number of Strains	Type of illness	Where isolated	Reference
1	Pneumonia	Copenhagen, Denmark	Kauffmann (1935)
1	Undescribed, but organism was isolated from blood.	Shanghai, China	Kauffmann (1941)
4	Paratyphoid infection, ulcer, healthy carrier	Philippines	Stevens (1946)
1	Gastro-intestinal and upper respiratory symptoms	New Orleans, U.S.A.	Holt and Newton (1948)
1	Typhoid-like fever with erythema	New Orleans, U.S.A.	Holt and Newton (1948)
5	Typhoid-like fever with focal suppuration, or abscess formation only	New Guinea	Baker and Bragdon (1947)
34	Mainly enteric-like fever sometimes complicated by local suppuration, one gastro-enteritis case	New Guinea and Bougainville Island	Atkinson, Woodroofe and Macbeth (1944, 1947, 1949); present paper
1	Enteric-like fever with cystitis	New South Wales, Australia	Fenner and Jackson (1946); Jones and Fenner (1947)
1	Epidemic disease in guinea-pigs	Victoria, Australia	
6	Epidemic disease in mice	Victoria, Australia	

The majority of strains, namely 39, have been found in infections occurring in New Guinea; but a few strains have been recorded from cases in Denmark, China, the Philippines, U.S.A. and Australia. With the possible exception of the one Australian case no evidence appears in the literature to suggest that these latter cases contracted their infection in New Guinea. However the prevalence of the organism seems undoubted in this locality in which 72 cases of infection (not all bacteriologically proven) have been recorded (Fenner and Jackson, 1946, Baker and Bragdon, 1947, Jones and Fenner, 1947) as compared with one or two cases only in Europe, Asia, America or Australia.

S. blegdam is pathogenic for guinea-pigs and mice and the only natural epidemic recorded in these animals provided the strains described by Atkinson, Woodroofe and Macbeth (1946). This epidemic was mentioned by Fenner and Jackson (1946). Mr. E. L. French who observed the epidemic and isolated the cultures stated in a personal communication to us that no connection between these animals and any materials from New Guinea could be traced nor were any cases of *S. blegdam* present at the time in the hospital which his laboratory served. We readily infected mice, guinea-pigs or young rabbits with representative strains from our group of smooth *S. blegdam* cultures. Intraperitoneal inoculation of these animals with 0·25 to 0·5 ml. of a 24 hours' broth culture usually resulted in death in 24–72 hours. Post-mortem examination revealed no obvious changes in internal organs and *S. blegdam* was recovered from the heart blood. Mice or guinea-pigs receiving smaller inocula and surviving for five or more days frequently showed small whitish lesions in the liver from which *S. blegdam* was isolated. Sections of the liver of a guinea-pig dying 10 days after intraperitoneal inoculation of about 15,000 organisms (strain XLIX) showed marked infiltration of inflammatory cells (chiefly macrophages and lymphocytes with a few polymorphs) which were partly gathered into discrete foci and in part diffusely distributed; some of the hepatic cells showed degenerative changes but no definite necrotic foci were detected[2]. Guinea-pigs injected intramuscularly

[2] For this report we are indebted to Dr. I. W. McDonald who was, at the time, veterinary pathologist to the Institute of Medical and Veterinary Science.

with about 100 × 10^6 organisms developed an abscess at the site of injection from which pus containing *S. blegdam* was discharged.

In mice after intraperitoneal inoculation the survival time depended not only upon the size of the inoculum but also upon the strain of *S. blegdam* used. Some results are given in Table 4.

TABLE 4.

Virulence tests in mice with strains of S. blegdam.

Strain Number	Reaction with phage†	Dose* (in millions)	Number of mice injected	Number of mice surviving in 2 days	Number of mice surviving in 4 days
XLIX	+	120	4	0	0
		60	4	0	0
		30	4	0	0
N.II	—	120	4	4	1
		60	4	4	3
		30	4	4	4
XXIII	+	120	4	0	0
		60	4	3	1
		30	4	3	2
McCall	—	120	4	4	0
		60	4	4	4
		30	4	4	4

* The dose consisted of organisms washed off a 24 hours' agar slope, centrifuged out and then suspended in saline. The number of *S. blegdam* in the suspension was estimated by comparison with turbidity standards using the figures quoted for *S. typhi*. All injections were intraperitoneal.

† Details of phage preparations used and methods of testing are given in the section on investigations with phages.

The comparison of XLIX and N.II was carried out at a different time from that of XXIII and McCall. In the first experiment in Table 4 mice inoculated with N.II survived longer than those inoculated with XLIX, although all mice had died in 14 days. The second experiment in Table 4 showed longer survival of mice inoculated with McCall as compared with those receiving XXIII, but all except two McCall mice were dead in 14 days. Increase in virulence of N.II and McCall resulted from passage through mice. After a single mouse passage an intraperitoneal dose of 100 × 10^6 organisms of either of these strains killed mice in 24–48 hours. Increase in virulence correlated with increase in susceptibility to an adapted paratyphoid B Vi phage the tests with which are recorded in the next section.

Strain N.XIV which was rough was inoculated intraperitoneally into mice in doses of 500 × 10^6 organisms. The mice died in 24 hours and the organism was obtained from the heart blood. It was still rough. Serial passage through mice failed to render the organism smooth although it regularly killed the mice and was recovered from the blood.

Investigations with Phages.

Using the plate method recommended for Vi phage typing of *S. typhi* by Craigie and Yen (1938) a number of smooth strains of *S. blegdam* were tested for susceptibility to various phages kindly provided by Dr. J. Craigie and Dr. A. Felix to whom we are very grateful. The results of these tests are given in Table 5.

TABLE 5.

Results of tests on strains of S. blegdam *with typhoid and paratyphoid B Vi phages.*

S. blegdam Strain number	Typhoid Vi phages			Paratyphoid B Vi phages				
	I	II	IV	1	2	3a	$3a_1$	3b
XLIX	—	—	—	+	—	0	0	+
N.II	—	—	—	—	—	—	—	—
N.VII	0	—	0	+	—	—	—	+
N.VIII	—	—	—	+	—	—	—	+
F.M.	0	—	0	+	—	—	—	+
Bragdon	0	—	0	+	—	—	—	+
Seligmann A 4119	0	—	0	+	—	—	—	+
Seligmann B 4168	—	—	—	+	—	—	—	+
XIII	—	—	—	0	0	0	0	0
XXIII	—	—	—	0	0	0	0	0
XLV	—	0	—	0	0	0	0	0
Duncan	—	0	—	0	0	0	0	0
Jap. P.O.W.	—	—	—	0	0	0	0	0
N.I	—	—	—	0	0	0	0	0
LI	0	0	0	—	—	—	—	—
LII	0	0	0	—	—	—	—	—
N.XIV	0	0	0	—	—	—	—	—
S. blegdam	0	—	0	—	—	—	—	—

The phage preparations, with critical test dilutions between 10^{-3} and 10^{-6}, were used undiluted. Typhoid Vi phage II was represented by the types mentioned in the text.
— = no lysis detected. + = confluent or semi-confluent lysis. 0 = not tested.

No lysis of any strain of *S. blegdam* was detected with full strength typhoid Vi phages I or IV or any of the typing phages developed from typhoid Vi phage II including Craigie types A, C, E, F, G, H, J and L and Felix types A, C, D_1, D_2, D_3, D_4, D_5, E_1, E_2, F_1, F_2, G, H, J, L_1, L_2, M, T, 91 and Richmond.

With paratyphoid B Vi phages described by Felix and Callow (1943), lysis of the majority of strains of *S. blegdam* occurred with phage types 1 and 3b but not with phage types 2, 3a and $3a_1$. However, several strains (N.II, LI, LII and NXIV) showed no lysis with any phage. Of these, strain N.XIV was rough as already recorded. A series of dilutions of paratyphoid B Vi phage type 1 was tested in the same way on *S. blegdam* (strain XLIX) and *S. paratyphi B* types 1 and 2. On the homologous *S. paratyphi B* confluent lysis occurred to a dilution of 10^{-6} but only semi-confluent lysis appeared with undiluted phage on *S. paratyphi B* type 2 and *S. blegdam*.

Attempts were made to adapt typhoid Vi phages I and II to *S. blegdam* (strain XLIX) by the method of Craigie and Yen (1938) for adaptation of typhoid Vi phage to strains of *S. typhi*. After six to ten passages on *S. blegdam* the activity of the phage had greatly decreased or entirely disappeared. No adaptation to *S. blegdam* occurred and any remaining activity was directed only against *S. typhi*. Passage of *S. paratyphi B* Vi phage type 1 or type 3b, in the same way, on *S. blegdam* (strain XLIX) resulted in adaptation of the phages to *S. blegdam* as indicated by the greatly increased dilution causing lysis of this organism. After about six passages these phages produced confluent lysis on *S. blegdam* (strain XLIX) to a dilution of 10^{-6}. They also produced confluent lysis to the same dilution with the homologous type of *S. paratyphi B*. With the *blegdam*-adapted phages all strains of *S. blegdam* were tested. The results are given in Table 6.

The results in Table 6 indicate variation among the strains of *S. blegdam* in susceptibility to lysis by the blegdam-adapted phages. Thus strains XII, XIII, XIX, XLI, XLIII, XLV,

TABLE 6.

Results of tests on strains of S. blegdam *with blegdam-adapted paratyphoid B Vi phages types 1 and 3b.*

S. blegdam Strain number	Lysis before mouse passage		Lysis after mouse passage
	Phage type 1	Phage type 3b	Phage type 3b
VII	++	++	0
VIII	++	++	0
X	++	0	0
XII	—	±	++ (1)
XIII	—	—	++ (1)
XIX	—	—	++ (1)
XXIII	++	++	0
XXV	++	++	0
XXIX	++	++	0
XXX	++	++	0
XXXVIII	++	++	0
XXXIX	++	++	0
XLI	—	—	++ (1)
XLII	++	++	0
XLIII	—	—	0
XLV	±	±	++ (2)
XLIX	++	++	0
LI	—	—	++ (1)
LII	—	—	++ (1)
Gray	—	—	0
McCall	—	—	++ (2)
Duncan	—	—	++ (1)
N.I	++	++	0
N.II	—	—	++ (1)
N.V	++	++	0
N.VI	++	++	0
N.VII	++	++	0
N.VIII	++	++	0
N.IX	—	—	++ (1)
N.X	—	—	++ (1)
N.XI	—	—	++ (1)
N.XII	±	—	++ (1)
N.XIII	—	—	0
N.XIV	—	±	—
Jap. P.O.W.	++	++	0
Bragdon	++	0	0
Seligmann A 4119	++	++	0
Seligmann B 4168	++	++	0
F.M.	++	++	0
M.1	++	++	0
M.2	—	—	++ (1)
M.3	++	++	0
M.4	++	++	0
M.5	++	++	0
G.P.H.B.	++	++	0
S. blegdam	—	—	++ (1)

The phage preparations were used undiluted. The critical test dilution was 10–2.
++ = confluent lysis. ± = a few plaques. — = no lysis. 0 = test not done.
The figures in brackets represent number of mouse passages.

LI, LII, Gray, McCall, Duncan, N.II, N.IX, N.X, N.XI, N.XII, N.XIII, N.XIV, M2 and the type culture of *S. blegdam* were resistant to lysis or only slightly lysed by either phage. All these strains except N.XIV were smooth. By passage through mice many of these strains were rendered phage-susceptible as shown by the results in Table 6 for lysis after mouse passage. Of the 16 smooth strains tested in this way only two, XLV and McCall, required more than one mouse passage to effect the change. The rough strain N.XIV did not change with repeated mouse passages.

The reverse change from phage-susceptible to phage-resistant was induced in several strains by cultivation in the presence of the phage and subculture from the secondary growth appearing in the zone of lysis or in the lysed broth culture. The growth thus obtained was still smooth.

Indications of lowered virulence in phage-resistant strains of *S. blegdam* were obtained in preliminary tests with N.II in which mice inoculated intraperitoneally with about 100 × 10^6 organisms survived for at least three to four days. On isolation from the mice strain N.II was phage-susceptible and in a dose of 100 × 10^6 killed mice in 18 hours or less. The results already given in Table 4 show the reduced virulence of N.II as compared with a phage-susceptible strain XLIX. The other results in Table 4 also indicate lower virulence for McCall, a phage-resistant strain compared with XXIII a phage-susceptible strain. Futher evidence is presented in Table 7 from which elevated virulence is again indicated for the phage susceptible strains. All cultures were smooth by the trypaflavine test, and by colonial appearance. Further work on the relation between phage-susceptibility and virulence will be recorded in a separate paper.

TABLE 7.

Virulence tests in mice with strains of S. blegdam.

Strain Number	Reaction with phage	Dose (in millions)	Number of mice injected	Number of mice surviving after	
				18 hrs.	6 days
N.XIII	—	200	5	3	1
		100	5	3	0
		50	5	5	4
XIX (after mouse passage)	+	200	5	0	0
		100	5	0	0
		50	5	1	0
				36 hrs.	4 days
N.X	—	500	5	0	0
		100	5	3	0
		50	5	5	4
N.X (after mouse passage)	+	500	5	0	0
		100	5	0	0
		50	5	0	0

DISCUSSION.

S. blegdam, as represented by our group of 45 strains, gave the typical reactions of the Salmonellas. In peptone water it failed to ferment lactose, sucrose or adonitol but promptly produced acid and gas from glucose, maltose and mannitol. It did not liquefy gelatine or produce indol. According to Kauffmann (1941) H_2S is produced by the majority of Salmonellas and we obtained

abundant H_2S from *S. blegdam*. Kauffmann found variation among Salmonellas in fermentation of inositol and we have confirmed that *S. blegdam* does not attack inositol. With dulcitol, which Kauffmann recorded as promptly fermented by the majority of Salmonellas we found as he did that *S. blegdam* produced acid and gas only after three or four days.

In Bitter solution and on Simmons agar all our strains failed to attack dulcitol although Kauffmann obtained a slow positive reaction with his *S. blegdam* 1 with this substrate on Simmons agar. However, Kauffmann's *S. blegdam* 2, a variant obtained from the original strain 1, produced no positive reactions in Bitter or Simmons tests. Our rough strain N.XIV resembled *S. blegdam* 2 as it produced negative reactions in the Bitter tests and negative or slowly positive reactions on Simmons agar. According to Kauffmann, these tests are of some value in distinguishing between the closely related members of the enteritidis group. *S. enteritidis* differed from *S. blegdam* in giving rapidly positive tests in both Bitter solution and Simmons agar with dulcitol.

However, the only reliable criterion for distinguishing *S. blegdam* from other enteritidis types is the possession of the distinctive combination of H factors gmq. *S. enteritidis* possesses a closely related group of H factors gm and may lead to confusion. Careful analysis for antigen m and for antigen q allows the separation of the two types. If q is overlooked in examining a strain of *S. blegdam* it will be erroneously labelled *S. enteritidis*, an oversight which occurred in the original investigation of the Bragdon strain of *S. blegdam*. Obviously then the routine identification of an organism as *S. blegdam* rests upon the somewhat insecure basis of the result of a single serological test, the agglutination with absorbed antiserum for factor q. If the factor q were lost the organism would then become *S. enteritidis*. We are at present attempting to suppress q by passage of *S. blegdam* in semi-solid agar with specific q antiserum. So far q is still detectable after about a dozen passages. This work is continuing.

Thus the evidence upon which the identification of *S. blegdam* rests at present is rather slim. However, our investigations suggest that other differences exist between *S. blegdam* and *S. enteritidis* as, for example, in pathogenicity for man and in geographical distribution. In contrast to *S. enteritidis*, *S. blegdam* seldom causes gastro-enteritis in man in whom it mainly produces an enteric type of infection. The vast majority of cases of blegdam enteric fever occurred in New Guinea but *S. enteritidis* has been found causing food-poisoning all over the world. In India Hayes and Freeman (1945) recorded *S. enteritidis* as causing enteric-like fever with occasional complications of a suppurative type. They especially marked the slow dulcitol-fermentation of many of their strains. Wilson and Miles (1946) mention that occasionally strains identified as *S. enteritidis* have been found in enteric-like fevers of man but the accuracy of the identification of the organism may often be questioned and they suggest that

these strains may have been the dublin type. We suggest that enteritidis strains causing enteric-like fever may well prove to be *S. blegdam*. This organism can now take its place beside *S. typhi*, *S. paratyphi*, *A*, *B* and *C* and *S. choleraesuis* as giving rise to enteric fever, in which, like *S. choleraesuis*, it tends to produce complications due to suppurative lesions such as cystitis, pericarditis or cholecystitis.

In our work elevated virulence of *S. blegdam* for mice was associated with susceptibility to blegdam-adapted paratyphoid B Vi phage type 1 or 3b. Loss of susceptibility to the phage was not necessarily accompanied by rough variation as judged by colony form and the trypaflavine reaction. Lowered virulence also appeared in our naturally rough strain N.XIV which still however killed mice in doses comparable to those required with phage-resistant smooth strains. These results suggest that virulence for mice may be influenced not only by the S→R variation well recognized as affecting virulence in other organisms, but also by another variation resulting in alteration in susceptibility to the blegdam-adapted phage. These two variations, presumably in the antigenic or other material situated near the surface of the cell, may apparently occur independently as we have preliminary presumptive evidence for the presence in the rough strain N.XIV of the factor responsible for phage-susceptibility. Phage action on this strain was not obvious but by passage on it a paratyphoid B Vi phage has been adapted to *S. blegdam*. This adapted phage showed good lysis of strains susceptible to our other phage preparations and failed to lyse the resistant strains. An adapting factor similar to that present in smooth susceptible strains seems therefore to be present in this rough strain. This work suggests that the more certain differentiation of *S. blegdam* from *S. enteritidis* may depend upon demonstrating unrecognized differences in the somatic make-up of the two organisms.

SUMMARY.

Biochemical and serological reactions, Vi phage sensitivity and virulence of forty-five strains of *S. blegdam* have been studied. All strains gave the typical biochemical reactions for the Salmonella group. They also failed to ferment inositol and fermented dulcitol only after three days. Haemolysin, fibrinolysin and coagulase were not detected under various conditions. The organisms were identified by their typical flagellar antigens gmq.

Typhoid Vi phages failed to lyse any of our strains of *S. blegdam*.

Paratyphoid B Vi phage type 1 or 3b produced partial lysis with some strains but other strains proved resistant. The paratyphoid B Vi phages were easily adapted to *S. blegdam*. When tested with blegdam-adapted phage our strains fell into two groups, susceptible and resistant. The virulence for mice of phage-susceptible strains appeared greater than that of resistant strains. By mouse passage resistant strains were rendered phage-susceptible and at the same time their virulence for mice was increased.

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